Keratin 20 Serine 13 Phosphorylation Is a Stress and Intestinal Goblet Cell Marker*\(^{\text{F}}\)

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Keratin polypeptide 20 (K20) is an intermediate filament protein with preferential expression in epithelia of the stomach, intestine, uterus, and bladder and in Merkel cells of the skin. K20 expression is used as a marker to distinguish metastatic tumor origin, but nothing is known regarding its regulation and function. We studied K20 phosphorylation as a first step toward understanding its physiologic role. K20 phosphorylation occurs preferentially on serine, with a high stoichiometry as compared with keratin polypeptides 18 and 19. Mass spectrometry analysis predicted that either K20 Ser\(^{13}\) or Ser\(^{14}\) was a likely phosphorylation site, and Ser\(^{13}\) was confirmed as the phospho-moiety using mutation and transfection analysis and generation of an anti-K20-phospho-Ser\(^{13}\) antibody. K20 Ser\(^{13}\) phosphorylation increases after protein kinase C activation, and Ser\(^{14}\)-to-Ala mutation interferes with keratin filament reorganization in transfected cells. In physiological contexts, K20 degradation and associated Ser\(^{13}\) hyperphosphorylation occur during apoptosis, and chemically induced mouse colitis also promotes Ser\(^{13}\) phosphorylation. Among mouse small intestinal enterocytes, K20 Ser\(^{13}\) is preferentially phosphorylated in goblet cells and undergoes dramatic hyperphosphorylation after starvation and mucus secretion. Therefore, K20 Ser\(^{13}\) is a highly dynamic protein kinase C-related phosphorylation site that is induced during apoptosis and tissue injury. K20 Ser\(^{13}\) phosphorylation also serves as a unique marker of small intestinal goblet cells.

Keratins are intermediate filament (IF\(^{3}\)) cytoskeletal proteins that are preferentially expressed in epithelial cells. All IF proteins consist of a central \(\alpha\)-helical "rod" domain that is flanked by non-\(\alpha\)-helical N-terminal "head" and C-terminal "tail" domains. Expressions of unique complements of type I (keratin polypeptides 9–20 (K9–K20)) and type II (K1–K8) keratins, which associate at a noncovalent 1:1 ratio of type I to type II heteropolymers, distinguishes different epithelial subtypes. For example, keratinocytes preferentially express K5/K14 or K1/K10 depending on their differentiation state in the epidermis; adult hepatocytes express K8/K18 exclusively, whereas intestinal epithelial cells express K8 as the major type II keratin with varying levels of the type I keratins, K18/K19/K20, depending on the differentiation state (e.g. crypt versus villus cells) or cell type (e.g. goblet versus absorptive cell) (1–3). The functions of keratins (and other IF proteins) are becoming increasingly well understood (4–7) as IF-null or dominant negative mouse models are developed and studied (8) and as mutations in IF proteins are linked to a growing list of human diseases (9). These functions are mechanical and nonmechanical in nature and include providing tissue and cell integrity, protecting cells from apoptosis and nonmechanical forms of injury, tissue-specific functions, cell signaling, and maintenance of subcellular organelle positioning and functions.

Keratin and other IF protein functions are regulated by posttranslational modifications (2) and an increasingly appreciated and growing list of associated proteins (5, 10). In terms of posttranslational modifications, phosphorylation is the most studied (11–13) and occurs preferentially within the head and tail domains of keratins, which are the most polymorphic domains within IF proteins. Of the more than 20 keratins, only K1, K4–K6, K8, K18, and K19 have been studied in terms of their phosphorylation (11, 14–17). Of these keratins, the most extensively studied are K8 and K18, with several established generalized or site-specific roles for their phosphorylation that include: (i) serving as a marker of epithelial cell injury (18); (ii) protecting the liver in transgenic mice from toxic injury (19); (iii) protecting K18 from caspase-mediated degradation (20) and K8 from proteasomal degradation (21); (iv) regulating the interaction of binding partners such as 14-3-3 proteins, which in turn appears to have an effect on keratin filament organization in tissues and on regulating mitotic progression after partial hepatectomy in mice (22); and (v) regulating keratin filament reorganization in response to stimuli in cell culture systems (23). Thus, the understanding of keratin phosphorylation has provided a useful handle to study keratin function.

Among the epithelial keratins, K20 has several unique properties. When compared with all other type I keratins, K20 has greater sequence divergence with only 58% amino acid identity within the conserved \(\alpha\)-helical rod domain with the closest keratin, K14 (24). It has unique cell distribution that is nearly confined to the gastric and intestinal epithelium, urothelium, bladder, and Merkel cells (25, 26). Because of its restricted distribution, K20 has been widely used clinically as a marker to help assess the origin of metastatic tumors (27, 28). The biological role of K20 is unclear, although mating experiments with transgenic mice that overexpress wild-type or mutant K18 or K20 have clearly demonstrated that K20 provides filament organization functional redundancy in cells that express more than one type I keratin (3). In addition, the preferential expression of K20 in suprabasal villus cells (3) and its marked induction in acinar cells after pancreatic injury (29)
suggest that it may have differentiation or stress-related functions. As a step toward understanding K20 regulation and function, we have described here the characterization of K20 phosphorylation. We show that K20 Ser13 phosphorylation is: (i) increased upon protein kinase C (PKC) activation, (ii) induced during apoptosis and tissue injury, and (iii) serves as a unique marker of small intestinal goblet cells.

EXPERIMENTAL PROCEDURES

Cells and Reagents—HT29 (human colon), NIH-3T3 (mouse fibroblast), and BHK-21 (baby hamster kidney) cells were obtained from the American Type Culture Collection. BHK-21 cells do not express endogenous keratins (20, 21). Monoclonal antibodies (mAb) that were used included: L2A1 and DC10 (anti-human (h) K18), M20 (anti-hK8), ITKs20.10 (anti-hK20) (Neomarkers); mAb 4.62 (anti-hK19 (Sigma)); M30 (anti-caspase cleaved K18 (Roche Applied Science)). The antimucin antibody was kindly provided by Dr. Laurie L. Shekels (University of Minnesota). Other reagents include: phosphoric acid (32PO4; PerkinElmer Life Sciences); trypsin and chymotrypsin (Worthington); okadaic acid (OA; Alexis Biochemicals); phorbol 12-myristate 13-acetate (PMA; Sigma); anisomycin (Calbiochem). Apoptosis was induced in HT29 cells (80–90% confluent) by culturing them with anisomycin (10 μg/ml) for 18 h. Cells were also cultured in the presence of PMA (200 ng/ml) for 30 min. Whole cell lysates were isolated by solubilizing in 2% SDS-containing Laemmli sample buffer for one dimensional SDS-PAGE or in a urea-containing buffer for isoelectric focusing (IEF).

Antibody Generation and Characterization—Human K20 was expressed using a pET bacterial protein expression system, and partially purified K20 was used to immunize Balb/c mice. After fusion and screening, one clone (termed Q6) was chosen, subcloned, and propagated. In addition, a rabbit phospho (p)-epitope-specific antibody was generated commercially (Anaspec) against the peptide CFHRSLpSSSLQA, which recognizes the human and mouse pSer13-containing K20. The Cys residue (which is not part of the K20 peptide) was added to the peptide sequence to facilitate peptide coupling to keyhole limpet hemocyanin. Two rabbit antibodies, Ab 2667 and Ab 2668 were generated. Ab 2667 showed superior reactivity to the K20 phosphoepitope (not shown) and was the antibody used for all described experiments.

Mouse Strains—Balb/c and FVB/n mice were used. For the starvation experiment, mice were given water alone for 18 h followed by tissue harvesting. For the dextran sodium sulfate (DSS) treatment, 5% DSS added in drinking water for 7 days was replaced with regular water for 12 h before sacrificing. All animals received care according to standard accepted criteria and guidelines.

Keratin Immunoprecipitation, Dephosphorylation, and High Salt Extraction—HT29 cells were solubilized with 1% Nonidet P-40 or with 1% Empigen in phosphate-buffered saline containing 5 mM EDTA and a protease inhibitor mixture (Sigma). After pelleting, supernatants were used for immunoprecipitation with mAb L2A1 (anti-K18), 4.62 (anti-K19), or Q6 (anti-K20). Isoelectric focusing was done as recommended by the supplier of the apparatus (Bio-Rad). High salt extraction was carried out to partially purify the majority of cellular keratins (30). For keratin dephosphorylation, keratin immunoprecipitates (isolated from HT29 cells) were incubated with shrimp alkaline phosphatase (37 °C,
Phosphorylation of K20 in cultured cells. A, HT29 cells were metabolically labeled with $^{32}$PO$_4$ followed by solubilization and immunoprecipitation of the indicated keratins from the Empigen-solubilized fraction (KB/K19 (mAb 4.62); KB/K18 (mAb L21); K20 (mAb Q66)). Keratin precipitates were analyzed by SDS-PAGE and Coomassie staining followed by autoradiography. B, a $^{32}$PO$_4$-labeled K20 immunoprecipitate was analyzed by two-dimensional gels. Coomassie staining, and autoradiography, which allowed assignment of the K20 isoforms 1–5. C, $^{32}$PO$_4$-labeled K20 was isolated and subjected to phosphoamino acid analysis. The positions of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) were confirmed by ninhydrin staining using unlabeled phosphoamino acids. D, $^{32}$PO$_4$-labeled K20 was subjected to tryptic phosphopeptide mapping to identify the number of phosphorylated peptides. x = origin of sample loading.

1 h) followed by washing and then IEF/SDS-PAGE, gel transfer, and immunoblotting.

**Phosphoamino Acid Analysis and Peptide Mapping**—HT29 cells were metabolically labeled with $^{32}$P orthophosphate for 4 h (250 µCi/ml) followed by immunoprecipitation using anti-K20 mAb. After gel separation and Coomassie Blue staining, the K20 band was removed following by extraction of K20 by electroelution. $^{32}$P-Labeled K20 was subjected to acid hydrolysis and phosphoamino acid analysis (31). Phosphopeptide mapping of electroeluted $^{32}$P-labeled K20 was carried out by exhaustive trypsinization of isolated K20 followed by two-dimensional gel analysis using electrophoresis in the horizontal direction and chromatography in the second dimension (31).

**Phosphorylation Site Identification Using Mass Spectrometry**—HT29 cells were cultured in the presence of OA (1 µg/ml, 2 h) followed by immunoprecipitation using anti-K20 mAb, gel separation, and then in-gel digestion using trypsin. Briefly, gel pieces were destained in 25 mM NH$_4$HCO$_3$, 50% acetonitrile, reduced using 10 mM dithiothreitol in 25 mM NH$_4$HCO$_3$, alkylated using 50 mM iodoacetamide, and then digested overnight using 50 ng of modified porcine trypsin in 25 mM NH$_4$HCO$_3$. Peptides were extracted using 50% acetonitrile, 5% trifluoroacetic acid and then separated on an Ultimate chromatography system with a 75 µm × 15 cm Pepmap column (both from LC-Packings). Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile, and a gradient of 5–40% B over 30 min was employed for peptide separation. As peptides eluted they were introduced on-line into a QSTAR Pulsar mass spectrometer (Sciex/Applied Biosystems). One-second spectra of intact peptides were followed by automatic selection of the most intense eluting peptide for a 3-s fragmentation spectrum. Peak masses and fragment ion assignments were labeled according to the nomenclature of Biemann (32), and we searched for data using the search engine Mascot (Matrix Science) and then manually analyzed them to confirm assignments.

**Mutagenesis, Transfection, and Immunofluorescence Staining**—The human K20 cDNA was mutated to convert Ser$^{13}$ or Ser$^{14}$ to Ala using a QuickChange site-directed mutagenesis kit (Stratagene), and mutations were confirmed by DNA sequencing. Transfections into NIH-3T3 or BHK-21 cells were done using WT or mutant hK20 cDNA plus hK8 cDNA. When needed, OA (1 µg/ml, 2 h) was added just prior to harvesting. Immunofluorescence staining was carried out as described (30). For total K20 staining, we used the anti-K20 mAb ITKs20.10, because the Q6 mAb does not work well after methanol fixation (not shown). For quantification of filament reorganization, cells from each treatment were counted (~150 cells/treatment) and categorized as having an exclusive punctuate pattern (dots) or a mixed punctate/filamentous pattern (collapsed) or lacking any punctuate pattern (normal filaments).
Keratin 20 Phosphorylation

RESULTS

K20 Has Multiple Serine Phosphorylation Sites—As part of the initial biochemical characterization of K20 phosphorylation, we sought to generate monoclonal antibodies to K20. This was done by cloning human K20 cDNA into a bacterial expression system (see “Experimental Procedures”) and then using a partially purified hK20 protein as an antigen for mouse immunization. After fusion and hybridoma screening and selection (not shown), the Q6 clone was chosen because of its strong and specific affinity to hK20 as demonstrated by immunoprecipitation (Fig. 1A) and Western blotting (Fig. 1B). Association of K20 with its type II partner, K8, after Empigen solubilization, is weaker than K18–K8 association (compare lanes 2 and 4, Fig. 1A). The identity of the Q6-immunoprecipitated species (Fig. 1A, arrowhead in lane 4) was confirmed by microsequencing (not shown) and by immunoblotting using an independent commercial anti-K20 mAb (ITKs20.10) (Fig. 1A). Two-dimensional gel analysis of Q6 immunoprecipitates showed multiple charged isoforms that were recognized by mAb ITKs20.10, suggesting a high basal K20 phosphorylation state (Fig. 1B). Treatment of keratin immunoprecipitates with alkaline phosphatase collapsed all the spots that were separated by IEF into one unphosphorylated species (Fig. 1C). This indicates that the multiply charged K20 isoforms are likely generated by K20 phosphorylation.

An initial assessment of K20 phosphorylation was carried out by in vivo labeling of HT29 cells with $^{32}$PO$_4$ followed by immunoprecipitation of K20 and then analysis by two-dimensional gels, phosphoamino acid determination and tryptic phosphopeptide mapping. As shown in Fig. 2A, the specific activity of K20 phosphorylation is significantly higher when compared with K18 but similar to K19 and K8. The $^{32}$PO$_4$-radiolabeled species immunoprecipitated by the anti-K20 mAb (Q6) includes only the acidic isoforms of K20, as confirmed by two-dimensional gel analysis (Fig. 2B). Most of the K20 phosphorylation under basal conditions involves serines (Fig. 2C) and includes several tryptic phosphopeptides, which suggests multiple phosphorylation sites (Fig. 2D) also supported by Fig. 1C. The nearly exclusive serine phosphorylation of K20 is similar to other keratins (11). However, the presence of multiple phosphorylation sites under basal conditions suggests that K20 has multiple dynamic sites, which in contrast to other type I keratins for which phosphorylation has been characterized and that have only one major phosphorylation site (hK18 Ser$^{52}$ (33); hK19 Ser$^{35}$ (15)).

Identification of Ser$^{13}$ as a K20 Phosphorylation Site That Is Regulated by PKC Activation—We used mass spectrometry to identify K20 phosphorylation sites. The starting material for this analysis was K20 purified from OA-treated cells, in order to maximize phosphorylation at sites protected by protein phosphatase 2A inhibition. As shown in Fig. 3, this analysis suggested that K20 Ser$^{13}$ or Ser$^{14}$ is phosphorylated within the K20 trialpetic peptide $^{10}$(R)SLSSSLQAPVVSTVGMQR. Mutational analysis was then carried out by transfecting BHK-21 cells with K20 S13A or K20 S14A (together with the partner K8 to stabilize the transfected K20) (Fig. 4, A and B). Immunoblotting of two-dimensionally separated lysates from the transfected cells showed that mutation of K20 Ser$^{13}$, but not K20 S$^{14}$, ablated a major K20 acidic isoform (Fig. 4B). This provides a molecular confirmation of the mass spectrometry results and indicates that K20 Ser$^{13}$ is phosphorylated in cultured cells.

The sequence context of K20 Ser$^{13}$ (RXXS) suggests that it is a potential PKC substrate (34). We tested this hypothesis by treating HT29 cells with PMA (a PKC activator) followed by immunoblotting using mAb Q6. As shown in Fig. 4C, PMA enhanced K20 phosphorylation (based on the increases in relative intensity in spots 2 and 3).
Effect of K20 Ser\(^{13}\) Mutation on Keratin Filament Organization—Given that keratin phosphorylation can play a role in keratin filament organization (15, 23), we tested whether K20 Ser\(^{13}\) → Ala (S13A) has an effect on keratin filament assembly. Under basal conditions, co-transfection of K8 with K20 S13A into NIH-3T3 cells does not have a significant effect on keratin filament assembly as compared with K20 WT or S14A transfectants. However, okadaic acid treatment, which results in the rearrangement of keratin filament networks (35, 36), caused significantly more keratin filament reorganization in cells transfected with WT or S14A K20 as compared with K20 S13A (Table 1). These results indicate that K20 Ser\(^{13}\) phosphorylation is important in promoting the disassembly of keratin filaments.

Characterization of K20 Ser\(^{13}\) Phosphorylation Using a Phospho-epitope-specific Antibody—We used the synthetic K20 phosphopeptide (\(^8\)FHRS\(^{15}\)p\(^{16}\)SSLQA, see Fig. 4A), which includes pSer\(^{13}\) and is conserved in humans and mice, to immunize rabbits and generate a phospho-epitope-specific antibody. The generated antibody, Ab 2667 (see “Experimental Procedures”), manifested much higher reactivity toward phospho-K20 (pK20) after cells were cultured in the presence of OA, as confirmed by immunoblotting (Fig. 5A) and immunofluorescence staining (Fig. 6D). As anticipated for a phospho-epitope-specific antibody, Ab 2667 recognizes only the acidic K20 isoforms after separation by IEF (Fig. 5B). This antibody also recognizes pSer\(^{13}\) specifically, as confirmed by transfecting cells with WT, S13A, or S14A K20 followed by two-dimensional immunoblotting, which showed that reactivity of Ab 2667 is abolished only in the S13A K20 transfectants (Fig. 5C). The utility of Ab 2667 was verified further by showing that its reactivity with K20 increases after HT29 cells were cultured in the presence of PMA (Fig. 5D). Ab 2667 also recognizes the same conserved phospho-site in mouse K20, and testing its binding to mouse intestine indicated that small intestine K20 Ser\(^{13}\) is more phosphorylated as compared with K20 in the colon (Fig. 5E; note the relative levels of K20 versus phospho-K20 in the two tissues).

K20 Ser\(^{13}\) Is Phosphorylated during Apoptosis—Among the type I keratins (K9–K20), K15, K17, K18, and K19 are all caspase substrates and have been shown to be cleaved during apoptosis (20, 37, 38). All type I keratins share the caspase cleavage motif VEVD (Fig. 6A), which suggests that K20 is a likely caspase substrate. This was confirmed by culturing HT29 cells with anisomycin (an apoptosis-inducing agent, e.g. see Ref. 20), which generated the two expected K20 cleaved products, p26 and p23 (Fig. 6B). The two K20 fragments remain associated, probably because of head/tail to rod domain interaction or the remaining association with the non-caspase cleaved K8 as noted previously for K18 proteolysis during apoptosis (20). Stimulation with anisomycin induced significant hyperphosphorylation of K20 Ser\(^{13}\) (Fig. 6C), with hyper-phosphorylation occurring preferentially in preapoptotic cells, as determined by double staining using the M30 antibody (which recognizes cleaved K18 during late stages of apoptosis (20, 39)) and pK20 (Fig. 6E). Therefore, K20 Ser\(^{13}\) hyperphosphorylation occurs during the early stages of apoptosis but becomes less prominent during the late stages of apoptosis.

K20 Phosphorylation Is a Stress and Goblet Cell Marker in Mouse Intestine—To begin addressing the physiological relevance of K20 Ser\(^{13}\) phosphorylation, we first examined the distribution of this phosphory outcry in mouse intestine. Interestingly, K20 Ser\(^{13}\) phosphorylation is

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**TABLE 1**

Quantification of keratin filament reorganization after OA treatment of transfected NIH-3T3 cells

|                | Intact filaments | Collapsed network | Dots |
|----------------|------------------|------------------|------|
| WT K20         | 43               | 52               | 5    |
| S13A K20       | 70               | 23               | 7    |
| S14A K20       | 44               | 53               | 3    |

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**FIGURE 5.** Biochemical characterization of the anti-pK20 antibody. A, HT29 cells were cultured in the presence or absence of OA followed by analysis of the total cell lysate by immunoblotting using anti-K20 (Q6) or anti-pK20 (Ab 2667) antibodies. B, a mix of HT29 cell lysates from untreated and OA-treated cells were separated by two-dimensional gels followed by blotting with anti-K20 or anti-pK20 antibodies. Note that anti-pK20 recognizes only the acidic isoforms 3–6 of K20. C, BHK-21 cells were transfected with WT K8 and one of three K20 constructs: WT, S13A, or S14A. Three days after transfection, lysates were prepared followed by two-dimensional gel separation and then immunoblotting with the indicated antibodies. D, HT29 cells were cultured in the presence or absence of PMA followed by preparation of cell homogenates and then blotting with anti-K20 or anti-pK20 antibodies. E, homogenates of mouse small intestine (S.I.) or colon were immunoblotted with antibodies to K20 and pK20.
found preferentially in few epithelial K20\(^+\) cells of the small and large intestine (Fig. 7, a–c and g–i). The K20 pS13\(^+\) cells of the small intestine had goblet cell features that were confirmed by double staining of mouse small intestine using anti-mucin and anti-pK20 antibodies (Fig. 7, d–f).

In the colon, K20 pSer13-positive staining was also prominent in goblet cells, but it involved primarily the lumen-proximal cells (Fig. 7, g–i).

The near exclusive presence of K20 Ser13 phosphorylation in goblet cells of the small intestine suggested that it could be related to the presence or absence of mucin accumulation. We tested this hypothesis by the induction of mucin secretion via starvation. After mice were starved for 18 h, microvillus height decreased (not shown), but more importantly goblet cells appeared to have emptied their mucin contents (Fig. 8A) with formation of cup-like clear-colored areas (arrows in Fig. 8A, panel b). This was confirmed by musicarmine staining, which showed a dramatic decrease in mucin staining (Fig. 8B) in association with a dramatic increase in K20 Ser13 phosphorylation (Fig. 8C). Hence, K20 Ser13 phosphorylation correlates with mucin secretion in the small intestine; but we cannot exclude the possibility that it may also be related to a stress starvation signal of goblet cells independent of mucin secretion. The potential effect of stress on K20 Ser13 phosphorylation led us to test whether mouse intestinal injury induced by DSS, which induces colitis, has a similar effect on K20 phosphorylation. DSS-mediated tissue injury does significantly increase K20 Ser13 phosphorylation, as determined by immunoblotting (Fig. 8D) and immunofluorescence staining (Fig. 8E) of colonic tissues isolated from control and DSS-treated mice. This injury also enhances K20 protein levels (Fig. 8D), as noted previously in other keratins of several tissues after epithelial tissue injury (29, 40–42).

**DISCUSSION**

**General Features of K20 and Its Phosphorylation**—We used a combined analytical, biochemical, molecular, and immunologic approach to study the phosphorylation of K20, which has not been studied to date. The enrichment of phosphorylated starting material, via analysis of keratins isolated from OA-treated cells, allowed us to identify K20 Ser13 as a highly dynamic phosphorylation site and then to study its biological significance in cells and tissues under a variety of biologic contexts. In general terms, overall K20 phosphorylation is stoichiometrically more...
abundant as compared with K18 but is relatively similar to K8 and K19 (Fig. 1). K20 phosphorylation under basal conditions occurs preferentially on serine residues (Fig. 2) and in that context is similar to other keratins (11). However, K20 differs from other type I keratins, specifically K18 and K19, which co-exist with K20 in some cell types such as enterocytes, in that it appears to be phosphorylated on multiple dynamic sites under basal conditions (Fig. 2); in contrast, K18 (33) and K19 (15) have, for the most part, one major dynamic site.

Another feature of K20 (phospho- and nonphospho-), which was unmasked using the detergent Empigen that is highly effective in solubilizing keratins (43), is that K20 is significantly less stable than the other type I keratins, K18 and K19, in its ability to form complexes with the type II keratin, K8 (Fig. 1). This suggests that the interaction between K20 and K8 is relatively “loose,” which is supported by in vitro association measurements of purified K8/K18, K8/K19, and K8/K20 mixtures (44). Such differences may provide better accessibility for K20 to undergo posttranslational modifications. K20 now also joins other type I keratins (i.e. K15, K17, K18, and K19) as an apoptosis-associated caspase substrate (Fig. 6). The precise significance of specific keratin phosphorylation events during apoptosis is unclear, but a likely role is the facilitation of the necessary cytoskeleton breakdown and related keratin filament reorganization (2, 45). In addition, keratin phosphorylation in the case of K18, inhibits caspase-mediated degradation in vitro, which suggests that it may play other regulatory roles during apoptosis (20).

Induction of keratin hyperphosphorylation (discussed below) and keratin protein overexpression are common in previously described observations in many epithelial cell injury models, and were similarly noted herein for in K20 after chemically induced colitis (Fig. 8). In addition, K20 protein induction has been noted after pancreatic injury (29), and such keratin overexpression is also described for K19 and/or K8 and K18 after injury in the liver (40, 41), pancreas (29), and gallbladder (42). It is unknown whether the hyperphosphorylation and increased keratin levels are mechanistically interrelated. The mechanism of mouse keratin overexpression appears to be posttranscriptional in K8/K18 induction after griseofulvin-induced liver injury (41) but transcriptional in nature for K8/K18/K19 after caerulein-induced experimental pancreatitis (29).

**Features of K20 Ser\(^{13}\) Phosphorylation**—Several biochemical and physiological properties were defined for K20 Ser\(^{13}\) phosphorylation (Fig. 9). For example, K20 Ser\(^{13}\) phosphorylation is highly dynamic, because cell culture in the presence of OA resulted in its dramatic hyperphosphorylation (Fig. 5). Its location within the head domain of K20 (Fig. 9) is in line with the location of all reported IF phosphorylation sites within the head and/or tail domains (9, 11). K20 Ser\(^{13}\) phosphorylation appears to play a role in keratin filament reorganization in response to OA stimulation (Table 1), and similar effects were noted in other keratin phosphorylation sites such as K19 Ser\(^{18}\) (15) and K8 Ser\(^{23}\) (23) and in vimentin phosphorylation sites regulated by cAMP-dependent kinase (47).

Another important feature of K20 Ser\(^{13}\) phosphorylation is its utility as a goblet cell marker of mouse small intestine. Within the small intestine, the exclusive presence of K20 pSer\(^{13}\) within goblet cells (Fig. 7) suggests that this phosphorylation or related events could associate with mucin secretion. Indeed mucin release in response to starvation...
correlated with marked hyperphosphorylation of K20 Ser13 (Fig. 8), although a direct causal relationship still needs to be established. The restricted association of K20 Ser13 phosphorylation with goblet cells in the small intestine, but less predominantly so in the large intestine, likely reflects functionally related regional differences between intestinal goblet cells. For example, mouse aquaporin-9 expression in goblet cells of the colon and small intestine differs (48), and the response of goblet cells in these two tissues differs among different secretagogues (49). The potential importance of keratins in goblet (or any exocrine) cell secretion is unknown, but potential associations deserve mention. For example, keratin filaments are abundant and are prominent feature of the cytoplasmic cup that surrounds the secretory granules (50). Interestingly, colons of K8-null mice (which lack cytoplasmic keratins in goblet and most other enterocytes) have significantly larger goblet cells with more prominent mucin-containing granules as compared with their wild-type counterparts (51). It is therefore tempting to speculate that keratins and their regulation (e.g. via phosphorylation) may play a role in some cases of exocrine cell secretion.

An additional feature of K20 Ser13 phosphorylation is its association with tissue injury (Figs. 8 and 9) and apoptosis (Fig. 6) as demonstrated by its hyperphosphorylation in response to DSS-induced colitis in mice and to anisomycin in cultured cells, respectively. The association of keratin phosphorylation with cell injury and apoptosis is a uniform finding that is seen in various tissue culture models (11, 52, 53) and in patients with liver disease (18, 54). However, specific phosphorylation

![Figure 8](image)

**FIGURE 8.** Histochemical staining and immunoblot analysis of mouse intestine after starvation and DSS treatment. A and B, ilea were isolated from control-fed and starved mice followed by fixation and then hematoxylin and eosin (A) and mucicarmine (B) staining as described under “Experimental Procedures.” L = lumen; arrows indicate goblet cells that have emptied their cargo; arrowheads point to red mucin staining of goblet cells. C, ileal tissue was isolated from four control-fed (C1–C4) and four starved (S1–S4) mice in two independent experiments (C1, C2 and S1, S2 in Experiment 1; C3, C4 and S3, S4 in Experiment 2). Tissue homogenates were prepared followed by immunoblotting using anti-K20 (Q6) or anti-pK20 (Ab 2667) antibodies. A tubulin immunoblot is also shown as a protein loading control. D, colons from three control (C1–C3) and three DSS-treated (D1–D3) mice were tested by immunoblotting as described in C. E, colon K20 and pK20 staining of untreated control and DSS-treated mice. Note that pK20 staining increases significantly after DSS treatment, but this increase occurs in non-goblet epithelial cells.

![Figure 9](image)

**FIGURE 9.** Schematic summary of K20 Ser13 phosphorylation and its biological significance. K20 (and all other IF proteins) is divided into three domains: a central coil-coil α-helix that is flanked by two non-α-helical N-terminal head and C-terminal tail domains. K20 Ser13 phosphorylation can be mediated by PKC and other potential kinases. K20 pSer13 serves as a marker for tissue injury and for goblet cells in the small intestine. This phosphorylation also takes place in the early stages of an apoptotic stimulus and appears to be important in allowing K20-containing keratin filaments to reorganize.
sites may become hypophosphorylated during apoptosis, as noted for K18 pSer13 in mice livers exposed to Fas (53). Overall, the characterization of K20 Ser13 phosphorylation and generation of the phospho-specific antibody that allowed linkage of this site to several relevant biological and physiological features highlight the importance of using a multipronged approach to the study of keratin posttranslational regulation.

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