Keratin 8 phosphorylation by p38 kinase regulates cellular keratin filament reorganization: modulation by a keratin 1-like disease-causing mutation

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ABSTRACT

Keratin 8 (K8) Serine-73 occurs within a relatively conserved type-II keratin motif (68NQSLPL), and becomes phosphorylated in cultured cells and organs during mitosis, cell stress, and apoptosis. Here we show that Ser73 is exclusively phosphorylated in vitro by p38 mitogen-activated protein kinase. In cells, Ser73 phosphorylation occurs in association with p38 kinase activation, and is inhibited by SB203580 but not by PD98059. Transfection of K8 Ser73→Ala or K8 Ser73→Asp with K18 generates normal-appearing filaments. In contrast, exposure to okadaic acid results in keratin filament destabilization in cells expressing wild-type or Ser73→Asp K8, while Ser73→Ala K8-expressing cells maintain relatively stable filaments. p38 kinase associates with K8/18 immunoprecipitates, and binds selectively with K8 using an in vitro overlay assay. Given that K1 Leu159→Pro (156NQSLPLLQPL→156NQSLQPL) leads to epidermolytic hyperkeratosis, we tested and showed that the analogous K8 Leu71→Pro leads to K8 hyperphosphorylation by p38 kinase and in transfected cells, likely due to Ser70 neo-phosphorylation, in association with significant keratin filament collapse upon cell exposure to okadaic acid. Hence, K8 Ser73 is a physiologic phosphorylation site for p38 kinase, and its phosphorylation plays an important role in keratin filament reorganization. The Ser73→Ala-associated filament-reorganization defect is rescued by a Ser73→Asp mutation. Also, disease-causing keratin mutations can modulate keratin phosphorylation and organization, which may affect disease pathogenesis.

Abbreviations: Ab, antibody; An, anisomycin; Emp, Empigen BB; IF, intermediate filament(s); K, keratin; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MMS, methyl methanesulfonate; NP40, Nonidet P40; OA, okadaic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; pS, phospho-serine; SAPK, stress-activated protein kinase; WT, wild-type.
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INTRODUCTION

The “soft” mucosal keratins (K)\(^1\) make up the intermediate filament (IF) proteins that are preferentially expressed in epithelial cells that line the inner and outer surfaces of animal tissues. These mucosal keratins consist of a large family (at least 20 members termed K1-K20) of cytoplasmic proteins, that are divided into relatively acidic type I (K9-K20, pI < 6), and relatively basic type II (K1-K8, pI ≥ 6) keratins (1-4). Epithelial cells generally express two or more keratin noncovalent heteropolymers in a 1:1 molar ratio of type I to II IFs, with an epithelial cell type-specific unique keratin complement. For example, single layered “simple-type” epithelia express K8 and K18, with variable levels of K19 and K20 depending on the cell type, while keratinocytes express K5/14 or K1/10 basally and suprabasally, respectively. The prototype structure of all IF proteins, including keratins, consists of a central coiled-coil \(\alpha\)-helix domain termed the “rod” that is flanked by non-\(\alpha\)-helical N-terminal “head” and C-terminal “tail” domains (5,6). Notably, the head and tail domains of keratins contain most of the structural heterogeneity among IF proteins and also include the domains that undergo phosphorylation. This and other accumulating data strongly suggest that phosphorylation plays an important role in regulating the tissue-specific functional roles of the large keratin family (7-11).

Although spectacular gains have been made in linking 14 of the more than 20 keratins to a number of skin, oral, esophageal and liver diseases (12-17), full appreciation of keratin and other IF protein function has been lagging. For some keratins, one clearly delineated function is to protect cells from mechanical and nonmechanical forms of injury, but how this occurs remains poorly understood (11,12,18,19). Regardless, an intact keratin filament network and how keratin
filaments are organized appear to be important effectors of this ability to maintain cellular integrity. This is borne out by many in vitro studies that correlated the importance of various keratin domains to form typical-appearing filaments and by the phenotypes that have been observed in patients with keratin diseases and in animal models that express different keratin mutants (11,13,15-17,19,20). While perturbations within the highly conserved proximal and distal ends of the rod domain [which harbor most of the described disease-causing keratin mutations but lack any evidence of phosphorylation (10,15)] have significant effects on filament organization in vivo and in vitro, keratin phosphorylation within the head and tail domains also plays a significant role in filament organization in vitro (8,21) and in vivo (9,10). In addition, keratin mutations within the head domains, that may modulate keratin phosphorylation, have been described. For example, mutations have been described that either introduce a new potential phosphorylation site [e.g. K1 $^{156}$NQSSLLQP→$^{156}$NQSSPLQP which renders Ser158 a potential proline-directed kinase phosphorylation site (22)] or remove possible phosphorylation sites (e.g. ref. 23).

Keratin phosphorylation has been most extensively studied in K8/18/19 (10), due in part to the relative solubility of these keratins as compared with epidermal keratins (24). These studies resulted in the identification of several phosphorylation-mediated K8/18 functions. For example, K18 Ser33 phosphorylation regulates keratin binding to the 14-3-3 family of proteins during mitosis, which in turn plays a role in keratin filament organization and solubility (25,26). A direct role for keratin phosphorylation may also occur, as noted for K19, whereby mutation of its major phosphorylation site (Ser35→Ala) altered keratin filament organization in transiently transfected cells (27). In addition, transgenic mouse studies showed that K18 Ser52
phosphorylation facilitates a protective role against hepatotoxic injury (28), a finding that has provided direct evidence for a number of correlative data that document increased keratin phosphorylation in association with a variety of stresses in cultured cells and in intact animals (29). In the case of human K8, three major in vivo phosphorylation sites have been identified: Ser23, Ser431, and Ser73. Ser23 is a highly conserved site among all type II keratins, which suggests a common keratin function for this modification, while Ser431 is a basally phosphorylated site that increases its phosphorylation specific activity during mitosis and upon exposure to epidermal growth factor in association with filament reorganization (30). In contrast K8 Ser73 phosphorylation behaves like an on/off switch in cultured cells and in tissues, with phosphorylation being “on” during mitosis, a variety of cell stresses including heat and drug exposure, and during apoptosis (31).

Although the function of K8 S73 phosphorylation was unknown, our hypothesis prior to embarking on this study was that its phosphorylation is likely to be important due to its on/off property and its association with important cell processes. Here we show that the mitogen activated protein kinase (MAPK) p38 (reviewed in ref. 32-35) is a physiologic kinase for K8 S73 phosphorylation, and demonstrate that K8 S73 phosphorylation plays a significant role in keratin filament reorganization in response to the phosphatase inhibitor okadaic acid. Since K8 Ser73 is proximal to a human disease mutation site in epidermal K1 (NQSLQPL→NQSPQPL, ref. 22; with K8 S73 being part of the motif 68NQSLQPL of K8), we generated the equivalent K1 mutation in K8 (i.e. NQSSLQPL→NQSSPLQPL) and showed that it increased K8 phosphorylation, as compared with wild-type K8. This skin disease-causing mutation also resulted in significant keratin filament collapse in the presence of okadaic acid. Therefore, K8
S73 phosphorylation plays an important role in modulating keratin filament reorganization. In addition, this is the first demonstration that human keratin disease-causing mutations can indeed result in keratin hyperphosphorylation, and that such hyperphosphorylation can affect keratin filament organization, which in turn may contribute to disease pathogenesis.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents: The antibodies (Ab) used were: L2A1 mouse monoclonal antibody (mAb) which recognizes human K18 (24); mAb LJ4 which recognizes K8 phospho(p)S73 (31); mAb 5B3 which recognizes K8 pS431 (30); rabbit Ab 8250 which recognizes K18 pS33 (26); rabbit Ab 3055 which recognizes K18 pS52 (36); mAb M20 (NeoMarkers; Freemont, CA) which recognizes K8; anti-Flag antibody (Sigma; St. Louis, MO). Other reagents used were: anisomycin (An), Empigen BB (Emp), p42 kinase, c-Jun amino-terminal kinase (JNK), and p38 kinase (Calbiochem-Novabiochem. Corp.; La Jolla, CA); methyl methanesulfonate (MMS) (Aldrich, Milwaukee, WI); PD98059, and anti-p38 and anti-phospho-p38 antibodies (New England Biolabs Inc.; Beverly, MA). SB203580 (kindly provided by Dr. John Lee, SmithKline Beecham Pharmaceuticals, King of Prussia, PA); orthophosphate ($^{32}$PO$_4$) and $\gamma$$^{32}$P-ATP (NEN Life Science Products, Wilmington, DE).

Cell Culture: HT29 (human colon), BHK (hamster kidney), and NIH-3T3 (mouse fibroblast) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended by the supplier. In order to activate p38 kinase, cells were incubated with An (10 $\mu$g/ml, 0-20 h) or with MMS (0.1 or 1 mg/ml, 0-24 h). Cells were then solubilized with 2% SDS-containing sample buffer (37) followed by shearing of the DNA with a 27 G needle then boiling for 2 min to generate a total cell lysate. Alternatively, cells were processed for
immunoprecipitation as described below. For the kinase inhibitors SB203580 (p38 kinase) and PD98059 (MAPK kinase), cells were pre-incubated with these compounds (20 µM and 100 µM, respectively) for 1 h, then treated with An for 2 h.

**Immunofluorescence Staining:** Transiently transfected cells were grown on cover slips and fixed 3 d after transfection, using 100% methanol (-20°C) for 3 min. Staining was done as described (26). For okadaic acid (OA) treatment, OA (1 µg/ml) was added to the transfected cells for 2 hours before fixation and processing. Fluorescence was analyzed using a Bio-Rad MRC1024 confocal laser scanning and a Nikon TE300 inverted microscope. Cells co-transfected with WT K18 and one of the four K8 constructs (WT, S73A, S73D, or L71P) were scored, after treatment with OA, based on their filament organization as: (i) cells with residual filaments, (ii) cells with fine dots but without any residual filaments, and (iii) cells with large dots.

**Cell Transfection and cDNA Constructs:** The K8 mutants K8 Ser73 → Ala (S73A), S73D and L71P were generated using a Tranformer™ mutagenesis kit (Clontech Laboratories Inc., Palo Alto, CA) as recommended by the supplier. Wild-type (WT) K8, WT K18, or mutant K8 cDNA’s were subcloned into the pMRB101 mammalian expression vector under control of the hCMV promoter. The Flag-tagged α isoform of WT p38 or p38 AF (kinase-inactive form due to double mutation at the phosphorylation sites, T180A and Y182F; ref. 38) were used to overexpress the p38 proteins in BHK cells with keratin constructs. Transient transfections into NIH-3T3 or BHK cells were done using LipofectAMINE as recommended by the supplier. The NIH-3T3 cells were used for immunofluorescence experiments since they provided a well-formed keratin filament-staining pattern, while BHK cells were used to generate keratins for the biochemical experiments since they had a higher transfection efficiency.
Biochemical Methods: Immunoprecipitation was carried out by solubilizing cells with 1% Emp (1 h, 4°C) in buffer A [phosphate buffered saline (PBS) (pH 7.4) containing 5 mM EDTA, 0.1 mM phenyl methanesulfonyl fluoride, 10 µM pepstatin, 10 µM leupeptin, 25 µg/ml aprotinin and 1 µg/ml OA], or by solubilizing cells with 1% NP40 in buffer A. After pelleting (15 min; 16,000g), keratins were immunoprecipitated from the supernatant using Sepharose-conjugated L2A1 followed by washing, analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (37) then staining with Coomassie blue. For immunoblotting, gels were transferred to membranes followed by blotting (39) with individual anti-keratin antibodies. Bound antibodies were visualized with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Two dimensional chymotryptic phosphopeptide mapping was carried out exactly as described (30,40) using electrophoresis in the first (horizontal) dimension and chromatography in the second (vertical) dimension.

The overlay assay was performed as described (41) with minor modifications. Briefly, total lysate and K8/18 immunoprecipitates from HT29 cells were analyzed using SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane (4 °C). The membrane was blocked with 3% BSA in PBS for 2 d, followed by incubation with 1 µg/ml of p38 kinase in PBS with 0.05% Tween and 0.1% BSA for 2 h (22 °C). After washing, the membrane was incubated with anti-p38 antibody for immunoblotting.

In vivo and in vitro ³²P-Labeling: In vitro kinase reactions were carried out using K8/18 immunoprecipitates. For each of the kinases used (p38, p42 and Jun kinases), the buffers provided by the supplier were used as recommended. Immunoprecipitates of K8/18 were washed two times with the respective kinase buffer (in addition to the routine washings as part of
immunoprecipitation), then incubated with 5 µCi of and γ-32P-ATP, the kinase, and 20 µM ATP (10 min in a total volume of 25 µl). The kinase reaction was quenched by adding 4 times the normal concentration of Laemmli sample buffer, followed by boiling for 90 seconds then analysis by SDS-PAGE and autoradiography. Metabolic labeling with 32P-orthophosphate was done by incubating cells (in 100 mm dishes) with 5 ml of phosphate-free Dulbecco’s Modified Eagles medium containing 10% dialyzed fetal calf serum and 100 mM glutamine for 30 minutes followed by the addition of 50 µl of normal medium and 250 µCi/ml 32P-orthophosphate. After labeling for 5 hours, keratins were immunoprecipitated from the detergent-solubilized cells using mAb L2A1 then analyzed by preparative SDS-PAGE and Coomassie staining, followed by isolation of the individual keratin stained bands for peptide mapping.

RESULTS

Examination of K8 S73 phosphorylation by mutational analysis and by in vitro phosphorylation:

We previously identified K8 S73 as a K8 phosphorylation site using what we termed a “reverse immunologic” approach (31). This was aided by an antibody termed LJ4, which was generated by immunizing mice with keratins that were purified from okadaic acid treated HT29 cells. As previously shown (31), and exemplified in Fig. 1A, mAb LJ4 selectively recognizes the hyperphosphorylated and slightly slower migrating K8 species, termed HK8. The HK8 species are present in very small amounts in exponentially growing HT29 cells as determined by immunoprecipitation with mAb L2A1, which recognizes the entire keratin pool (31), but become markedly enriched after immunoprecipitation with mAb LJ4. The LJ4 Ab recognizes HK8 exclusively (Fig. 1B, lane 1), and its reactivity is abolished if Ser73 is mutated to an alanine
(S73A) (Fig. 1B, lane 2). However, LJ4 does recognize K8 S73D weakly (Fig. 1B, lane 3), which migrates slightly faster than HK8 and a bit slower than K8, such that LJ4 has almost equal binding intensity to the barely visible Coomassie-stained HK8 as compared to the strongly staining K8 S73D species (Fig. 1B).

We compared the in vitro phosphorylation of K8 by the proline-directed MAPK's p38 and p42, given the sequence context of K8 S73 (71LLSPL) and the previous observation that K8 S73 becomes phosphorylated during heat stress and apoptosis (31). As shown in Fig. 1C, p38 kinase generates the radiolabeled HK8 species exclusively (a signature of S73 phosphorylation) while p42 kinase generates phosphorylated K8 and HK8 (K8>HK8; compare lanes 2 and 3). Mutation of the two major K8 phosphorylation sites, S23 and S431 (30), did not affect formation of HK8 upon in vitro phosphorylation of K8/18 precipitates with p38 kinase (Fig. 2A, lanes 2 and 4). In contrast, mutation of K8 S73 abolished formation of the HK8 species and resulted in barely detectable K8 phosphorylation (Fig. 2A, lane 3) that is likely due to S431 phosphorylation (the only other K8 potential proline-directed kinase site, with the sequence 429LTSPG). The specificity of p38 kinase towards K8 S73 is evident by the minimal formation of HK8 by p42 kinase (Fig. 2B), and the nearly equal generation of phospho-K8 and HK8 species by JNK (Fig. 2C). K8 S23, which is a major basally phosphorylated K8 site (30), is not phosphorylated in vitro by any of the three tested MAPKs, while K8 S431 phosphorylation occurs by p42 and JNK but not by p38 kinase (Fig. 2). Hence, the in vitro kinase assays of WT and mutant K8 immunoprecipitates indicate that both JNK and p42 phosphorylate K8 S431 and S73 relatively promiscuously, albeit to varied levels, in marked contrast to the selectivity of p38 kinase to the K8 S73 site. In addition, phosphorylation of K8 S73 does not appear to impact on K8 S431
Evidence of in vivo K8 S73 phosphorylation by a p38-like kinase: Given the findings in Fig. 1 and 2, we explored the role of p38 kinase as a potential in vivo K8 kinase by utilizing known specific activators and inhibitors of p38 kinase and by comparing phosphopeptide maps of in vivo versus in vitro p38-phosphorylated K8. As shown in Fig. 3A, activation of p38 kinase in cultured HT29 cells by anisomycin (An) (42), as determined by p38 phosphorylation, is associated with rapid K8 S73 phosphorylation. Similarly, the alkylating agent MMS, a known p38 kinase and JNK activator (43), generates the HK8 species in a dose and time dependent fashion (Fig. 3B). Inhibition of An-induced p38 kinase activation with the specific inhibitor compound SB203580 abrogated K8 S73 phosphorylation (Fig. 3C). In contrast, inhibition of ERK1/2 kinase activation with compound PD98059 did not significantly affect K8 S73 phosphorylation, but did inhibit K8 S431 phosphorylation as determined by blotting with mAb 5B3 (Fig. 3D).

A comparison of the chymotryptic phosphopeptide maps of K8 and HK8 that are isolated from in vivo phosphorylated cells shows that HK8 differs from K8 by the presence of peptides 2-5 and by the absence of the peptide highlighted by an unmarked arrow (Fig. 4, panels a and b). Interestingly, the phosphopeptide profile of HK8 that is generated by in vitro phosphorylation of K8 with p38 kinase shows five major peptides (Fig. 4c) that co-migrate with peptides 1-5 that are isolated from in vivo labeled HK8. This is confirmed by mixing in vitro and in vivo labeled K8 (Fig. 4d) and by mixing in vivo labeled HK8 with p38-labeled K8 (not shown). The five peptides are generated by incomplete chymotryptic digestion (not shown). Taken together, these results suggest that a p38-like kinase is likely to be involved, in vivo, in K8 phosphorylation at...
p38 kinase associates with K8/18 and phosphorylates K8 S73 in vivo, and binds to K8 in vitro:

We further substantiated in vivo p38 phosphorylation of K8 S73, by comparing K8 S73 phosphorylation in BHK cells transfected with Flag-tagged human WT p38 or kinase-inactive p38 AF (Fig. 5A). The overexpressed p38α proteins are detected with anti-Flag and anti-human-p38 antibodies. As anticipated, p38 AF is not recognized by phospho-p38 antibody, and K8 S73 phosphorylation increases in BHK cells that overexpress WT but not AF p38 (Fig. 5A, lanes 1-3). In addition, WT and AF p38 kinases coimmunoprecipitate with K8/18 in transfected cells (Fig. 5A, lanes 5 and 6); arrowhead and arrows indicate degraded K8 or apoptotic K18 fragments (44), respectively. Co-immunoprecipitation of p38 with K8/18 was not observed in non-transfected cells (e.g. HT29 cells), which may be related to the high levels of p38 kinase in transfected cells and the transient/weak nature of the kinase-substrate interaction (not shown). The interaction of p38 kinase with keratins was also confirmed using an in vitro overlay assay. As shown in Fig. 5B, p38 kinase bound specifically to K8 but not to K18. Taken together, these results support the conclusion that p38 kinase associates with K8 and phosphorylates K8 S73 in vivo.

Effect of disease-related keratin mutations on keratin phosphorylation: K8 Ser73 is part of the sequence 68NQSLLSPL, a sequence that is identical in all type II keratins (except for the S73-equivalent residue which is substituted by Ala in K7, Gln in K1-3 and Thr in K4-6; ref. 31). Several of the mutations that have been described for epidermal keratins result in amino acid substitutions that potentially create a new, or remove a potential, phosphorylation site. Given the known impact of phosphorylation on keratin filament organization (9-11), it is possible that such
mutations could impact significantly on keratin filament organization and disease pathogenesis although such a possibility has not been formally tested for any such mutation. In order to address this, we focused on one such mutation [Leu159→Pro of K1 in a family of patients with epidermolytic hyperkeratosis (22)] that occurs in the highly conserved Ser73-containing domain of K8 (i.e. Leu71 within 68NQSLLSPL of K8), by using K8 as a model system (since K1 cDNA is not available). This mutation generates a potential new proline-directed kinase related site at Ser70 of K8 (Ser158 of K1). As shown in Fig. 6A, the K8 L71P mutation significantly increased K8 susceptibility to in vitro phosphorylation by p38 (compare lane 1 with 2) and p42 kinases (compare lane 3 with 4), but not by JNK (compare lane 5 with 6). The K8 L71P mutation also increased K8 phosphorylation in transfected cells after exposure to okadaic acid (Fig. 6B). This was confirmed by the presence of an HK8-like species in cells transfected with the K8 L71P but not with WT K8 as determined by Coomassie staining (Fig. 6B; compare lane 1 versus 2) and confirmed by immunoblotting with antibodies that recognize the total and phospho-K8 pools (Fig. 6B, lanes 3-6). No change was noted in K18 Ser 52 phosphorylation (Fig. 6B; lanes 9 and 10), which represents the major K18 phosphorylation site (10), thereby indicating specificity of the increased phosphorylation towards the mutant K8. Of note, the L71P K8 mutation inhibits binding of the LJ4 antibody to K8 (Fig. 6B, lane 8) thereby indicating that L71 is part of the antibody epitope. Therefore, disease-causing keratin mutations can indeed result in keratin hyperphosphorylation as modeled by the K8 L71P mutation in vitro and in vivo.

Effect of K8 S73→A, S73→D and L71→P mutations on keratin filament organization: We tested the effect of the K8 S73→A, S73→D, or L71→P mutations on K8/18 filament organization in transfected cells. Transient co-transfection of NIH-3T3 cells with WT K18 and
one of the four K8 constructs: WT K8, K8 S73A, K8 S73D, or K8 L71P followed by immunofluorescence staining of K8/18 showed a normal-appearing and an indistinguishable filament organization among the four K8 constructs [shown only for WT and L71P K8 in Fig. 7, panels a and e, respectively; with very similar profiles for K8 S73A and S73D (not shown)]. However, exposure of the transfected cells to okadaic acid unmasked significant differences in filament reorganization when comparing WT, or S73D K8 with S73A K8; or when comparing WT K8 with L71P K8 (Fig. 7). For example and as shown in Fig. 7, okadaic acid resulted in 42% and 41% of the cells maintaining residual filaments in WT and S73D K8 transfected cells, respectively, while 61% of the cells transfected with S73A K8 had cells with intact filaments (a total of 120-180 cells were counted in 3 independent experiments, p<0.05). Hence, S73D rescues the filament reorganization defect caused by the S73A mutation, likely due to the negative charge of the aspartate.

In the case of the L71P K8 mutant, nearly 10% of the cells with collapsed filament (after exposure to okadaic acid) had prominent large dots (Fig. 7f) while none of the cells transfected with any of the other K8 constructs manifested this phenotype. These large dots likely represent coalesced smaller dots since longer exposure of cells expressing WT K8 results in progression from a fine dot to a large dot pattern (not shown). Taken together, these data suggest that K8 S73 phosphorylation is associated with keratin filament destabilization and likely occurs to facilitate reorganization of the keratin filaments, while the patient-associated K8 L71P mutation results in an exaggerated keratin hyperphosphorylation response upon okadaic acid stimulation with consequent amplified destabilization of the keratin filament network.

**DISCUSSION**
**K8 S73 is a physiologic substrate for a p38 MAPK:** The temporal associations of K8 S73 phosphorylation, as determined by the signature formation of the HK8 species and by reactivity with mAb LJ4, suggests that a stress-induced kinase is responsible for its phosphorylation. We tested, in vitro, three candidate kinases that are members of the MAPK superfamily, namely JNK, p42 (ERK1), and p38 kinase. Of these kinases, only p38 kinase phosphorylated K8 S73 exclusively, based on HK8 formation (Fig. 1C and Fig. 2A), while JNK and p42 kinase resulted in preferential phosphorylation of K8 (due to K8 S431 phosphorylation) with some phosphorylation of the S73 site (Fig. 1C; Fig. 2B and C). Further support for a physiologic role of p38 kinase in S73 phosphorylation includes: (i) association of K8 S73 phosphorylation with states that activate p38 kinase (e.g. An and MMS exposure of cells, Fig. 3A and B), (ii) generation of a chymotryptic phosphopeptide pattern, upon in vitro phosphorylation of K8 with p38 kinase, that is very similar to the pattern of HK8 but not K8 in vivo phosphorylation (Fig. 4), (iii) inhibition of K8 S73 phosphorylation by the selective p38 kinase inhibitor SB203580, but not by PD98059 (Fig. 3C and D) which inhibits Erk1/2 kinase activation by inhibiting MEK1/2 kinases, (iv) p38 kinase association with K8/18 immunoprecipitates and phosphorylation of K8 S73 by p38 kinase in transfected cells (Fig. 5A), and (v) specific binding of p38 kinase with K8 using an overlay assay (Fig. 5B). Hence, our data strongly implicates K8 S73 as a physiologic substrate for p38 kinase and adds K8 to the few known likely physiologic substrates of p38 kinase which include MAPK activated protein kinase-2 and ribosomal S6 kinase-B (45).

Our assignment of K8 S73 as a physiologic substrate of a p38 kinase pertains in particular to p38α although other p38-like kinases may be involved given the growing list of related p38 kinases. The p38 kinase family [also called stress activated protein kinase-2 (SAPK-2)] has
several known members including p38α (SAPK-2α), p38β (SAPK-2β), p38γ (SAPK-3), SAPK-4, and p38δ (46). These kinases share nearly 60-75% sequence identity and have some differences in substrate specificity and in inhibition by pyridinyl imidazole compounds such as SB203580. In our case, we only tested the p38α kinase, which is known to be inhibited by SB203580. It is likely that more than one kinase does phosphorylate K8 S73 in vivo since such phosphorylation occurs during mitosis, a variety of cell stresses and apoptosis (10). To that end, p38 kinase activation is reported after a variety of apoptotic stimuli, and can also occur upon induction of proliferation as noted for B cells (47,48). In addition, Fas receptor stimulation of HT29 cells activates JNK selectively, rather than p38 kinase, and results in phosphorylation of K8 S73.

**Disease-causing keratin mutations may modulate keratin phosphorylation:** Several epidermal keratin mutations have been described at sites that may potentially introduce or remove a phosphorylation site and hence may affect disease pathogenesis by modulation of keratin phosphorylation upon the appropriate cell stimulation (12,13,15-17). However, this potential of mutation-associated modulation of phosphorylation has not been formally tested for any of these mutations. Given that one such mutation in K1 (L159P) occurs at the highly conserved and K8 S73-like motif [K8 L71 in 68NQSSLSPSPL is the equivalent Leu (bold lettering indicates conserved residues in all type II keratins)], we tested in K8 the effect of the K1-equivalent L71P mutation. This mutation resulted in K8 hyperphosphorylation, likely due to phosphorylation at the newly generated proline-directed kinase site in 68NQSSLSPSPL. Hyperphosphorylation of K8 L71P was confirmed in cultured cells after exposure to okadaic acid and in vitro by p38 kinase
phosphorylation (Fig. 6), and was associated with abnormal keratin filament reorganization (Fig. 7). Hence, our results support the conclusion that disease-causing keratin mutations can indeed generate abnormally phosphorylated keratins in a fashion that will predictably depend on the context of the mutation. At least in some cases, such modulation of keratin phosphorylation can alter keratin filament organization (Fig. 8) in response to physiologic and nonphysiologic hyperphosphorylating stimuli.

We used okadaic acid as a model system for the induction of generalized hyperphosphorylation, including the K8 Ser73 site that undergoes phosphorylation in the presence of OA (31), since we were not able to visualize with confidence enough mitotic cells in our transient transfection system (not shown). Of note, phosphatase inhibitors, such as okadaic acid and microcystin, are major hepatotoxins in animals (49-51) and in humans (52). Therefore, despite their generalized effects, the use of such compounds in cultured cells provides a relevant and sensitive filament reorganization model system.

**K8 S73 phosphorylation plays an essential role in keratin filament organization:** One unique feature of the K8 S73 phosphorylation site, as contrasted with other known K8 and K18 phosphorylation sites, is its near absolute on/off property while other phosphorylation sites manifest up/down modulation of a basal phosphorylation state depending on the stimulus (10). This on/off property and the reversible induction of this phosphorylation suggest important biologic role(s) for this modification that represents the convergence of several contexts (e.g. stress, apoptosis, and mitosis; ref. 31) that include p38 kinase activation and subsequent K8 S73 phosphorylation. One common feature for these differing biologic contexts is the observed keratin filament reorganization that is associated with these processes. The data presented herein
suggest a unique function for K8 S73 phosphorylation, which is to allow keratin filaments to reorganize. The evidence for this role is the absence of a keratin-assembly defect upon transient transfection of a K8 S73A mutant, but the unmasking of a phenotype upon exposure to OA-mediated hyperphosphorylating conditions (Fig.7). Furthermore, the K8 S73D mutation rescues the S73A phenotype thereby supporting the role of the phosphoserine moiety at that site. Hence the aspartate substitution mimics the phosphate of K8 pS73 biologically by rescuing the K8 S73A phenotype (Fig. 7) and biochemically by altering the migration pattern in SDS-PAGE gels from K8 to HK8-like (Fig. 1B).

Another unique feature of the K8 pS73 species is their distribution among various cellular compartments, as compared with other K8 and K18 species that are phosphorylated at other sites. For example, K8/18 are found in increasing abundance in the sequentially isolated cytosolic, NP40, Emp and then post-Emp solubilized fractions (10,25). Interestingly, the HK8 species are distributed nearly uniformly throughout these fractions while keratins that are phosphorylated on K18 S52 or K18 S33 are preferentially found in the cytosolic and NP40-containing fractions (10,26). This implies that keratin species that are typically cytoskeletal and insoluble (K8 S73 state) become reorganized in a fashion that is associated with p38 kinase activation (and phosphorylation at other keratin sites) to favor generation of the K8 pS73 state and distribution within the different cellular compartments in order to facilitate filament reorganization (Fig. 8).

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FIGURE LEGENDS

Fig. 1: In vitro K8 phosphorylation by p38 or p42 kinases, and mAb LJ4 reactivity with K8 S73 mutants: Panel A: K8/18 immunoprecipitates from HT29 cells were prepared using anti-K18 mAb L2A1 or anti-K8 pS73 mAb LJ4, followed by SDS-PAGE then staining with Coomassie blue. Note that mAb LJ4 preferentially recognizes the K8 S73 phosphorylated species, HK8 (residual presence of K8 in lane 2 reflects the tetrameric nature of keratins that may contain two K18, one K8 and one HK8 molecules per tetramer). Panel B: BHK cells were co-transfected with WT K18 and WT K8, or with WT K18 and the indicated K8 phosphorylation mutants. K8/18 were precipitated with mAb L2A1, then analyzed by SDS-PAGE and Coomassie staining. Duplicate K8/18 immunoprecipitates were also separated by SDS-PAGE then blotted with mAb LJ4. Note that LJ4 reactivity is abolished in the S73A mutant, and is limited when blotted against the S73D mutant as compared with WT K8 (see text). Panel C: In vitro kinase assays were performed using K8/18 immunoprecipitates that were obtained from HT29 cells. Precipitates were incubated with 5 μCi of [γ-32P]-ATP, 20 μM ATP and 1 unit of p38 or p42 kinases.
kinase for 15 minutes followed by quenching with sample buffer then SDS-PAGE analysis, Coomassie staining and autoradiography.

**Fig. 2: Phosphorylation of WT K8 and K8 mutants by p38 kinase, p42 kinase or JNK:** BHK cells were co-transfected with WT K18 and WT K8, or with WT K18 and one of three K8 phosphorylation mutants (S23A, S73A or S431A). Three days after transfection, K8/18 immunoprecipitates were obtained then used in an in vitro phosphorylation assay with the indicated kinases. Precipitates were analyzed by SDS-PAGE, Coomassie staining then autoradiography.

**Fig. 3: Modulation of K8 S73 phosphorylation by activation or inhibition of p38 kinase:** Panel A: HT29 cells were treated with 0.1% DMSO (0 h time point) or with anisomycin (An, 10 µg/ml) for the indicated times. Total lysates were then prepared by solubilizing with SDS sample buffer. Lysates were separated by SDS-PAGE, transferred to membranes, then blotted with anti-p38 and anti-phospho-p38 kinase antibodies, or anti-K8 pS73 mAb LJ4. Panel B: HT29 cells were incubated with MMS then harvested after the indicated time points, solubilized with 1% NP40 followed by immunoprecipitation with mAb L2A1. K8/18 precipitates were separated by SDS-PAGE then stained with Coomassie blue or immunoblotted with mAb LJ4. Asterisks in lane 7 represent degraded K8 species. **Panels C and D:** HT29 cells were preincubated for 1h with 20 µM SB203580 (p38 kinase inhibitor) or 100 µM PD98059 (MAP kinase kinase inhibitor) followed by An treatment for 2h. K8/18 immunoprecipitates were obtained from 1% NP40 solubilized cells, then blotted with anti-K8 pS73 (mAb LJ4) or anti-K8 pS431 (mAb 5B3). K8 S431 phosphorylation is used as a control since we previously showed that this site becomes phosphorylated upon EGF stimulation and that it is likely to be phosphorylated in vivo by p42.
MAP kinase (30).

**Fig. 4:** Phosphopeptide maps of in vitro and in vivo phosphorylated K8 and HK8: HT29 cells were metabolically labeled with $^{32}$PO$_4$ (250 µCi/ml) for 5 hr (in the presence or absence of 100 µg/ml of MMS to generate the HK8 species) followed by immunoprecipitation of K8/18. K8 (from cells without MMS treatment, *Panel a*) and HK8 (from MMS-treated cells, *Panel b*) were individually isolated using preparative SDS-PAGE, followed by chymotryptic phosphopeptide mapping. Alternatively, K8/18 immunoprecipitates were obtained from untreated HT29 cells followed by in vitro phosphorylation using [$\gamma^{32}$P]-ATP and p38 kinase. K8 was separated by SDS-PAGE then subjected to chymotryptic peptide mapping (*Panel c*). Equal counts of the samples shown in panels a and c were also mixed and analyzed (*Panel d*). The “x” in the left lower corners indicates the origin where samples were spotted onto thin layer cellulose plates for 2-dimensional separation using electrophoresis (horizontal dimension) then chromatography (vertical dimension). Note that the bracketed spots 2-5, which are not phosphorylated in K8 in vivo, are phosphorylated in vivo in HK8 and are also generated after in vitro phosphorylation of K8 with p38 kinase. The K8 peptide highlighted by an unlabeled arrow becomes relatively dephosphorylated after MMS treatment in HK8 (compare a with b) and in K8 (not shown).

**Fig. 5:** Association of p38 kinase with K8/18 immunoprecipitates, and specific binding of p38 kinase to K8 in vitro: *Panel A:* BHK cells were co-transfected with WT K8/18 and one of the three constructs: vector, Flag-tagged WT or AF p38. Transfected cells were solubilized with SDS-containing sample buffer (total lysate) or with 1% NP40 followed by immunoprecipitation (i.p.) of K8/18. Total lysates and K8/18 precipitates were analyzed by SDS-PAGE and stained with Coomassie blue or transferred to PVDF membranes for immunoblotting with the indicated
antibodies. Panel B: Total lysate and a K8/18 immunoprecipitate were obtained from HT29 cells then separated by SDS-PAGE and transferred to a membrane. The membrane was incubated with purified p38 kinase, washed then blotted with anti-p38 antibody as described in Experimental Procedures.

Fig. 6: Effect of the L71P K8 mutation on K8 phosphorylation: Panel A: BHK cells were co-transfected with WT K18, and WT K8 or K8 L71P. After 3 d, cells were harvested followed by immunoprecipitation of K8/18. Precipitates were subjected to an in vitro kinase assay, followed by gel analysis and autoradiography as described in Fig. 1 legend. Panel B: Cells were transfected as in Panel A. Just before harvesting, cells were incubated with OA (1 µg/ml) for 2 h followed by immunoprecipitation of K8/18. Precipitates were analyzed by SDS/PAGE then Coomassie staining or were analyzed by immunoblotting using antibodies that recognize the total K8 pool, K8 pS73, K8 pS431, and K18 pS52. Note that the K8 L71P mutation results in hyperphosphorylation of K8 in vitro, using p38 kinase, and in transfected cells as determined by formation of the HK8 species.

Fig. 7: Immunofluorescence staining of NIH-3T3 cells transfected with WT or mutant K8: NIH-3T3 cells were co-transfected with WT K18 and one of the following K8 constructs: WT, S73A, S73D or L71P. Transfected cells were grown on cover slips, and 3 d after transfection they were further cultured in the presence (+OA) or absence (control) of okadaic acid (1 µg/ml) for 2 h. Cells were then fixed with methanol then stained using anti-K8 mAb M20. Note that OA results in the formation of a fine punctate pattern preferentially in WT and S73D K8 transfectants but less so in S73A transfectants. Also, note that OA-treated L71P K8 transfected cells form large keratin-staining dots that are not seen in the cells transfected with the other K8
constructs.

**Fig. 8: Proposed model for the significance of K8 Ser73 phosphorylation and the potential impact of disease-causing phosphorylation-modulating keratin mutations:** The exchange between the basal K8/18 filaments and the progenitor soluble filament pool is likely to be K8 S73 independent due to the absence of any detectable basal K8 S73 phosphorylation. Stimulation of cells, as may occur during cell stress or apoptosis, results in K8 S73 phosphorylation via p38 kinase and in “normal” keratin filament reorganization (with increased keratin solubility), which becomes limited upon a K8 Ser73→Ala mutation. A K8 Ser73→Asp mutation rescues the filament reorganization defect that is caused by blocking Ser73 phosphorylation. However, disease-causing mutations, such as the K1-like mutation that was introduced into K8 (L71P), can cause a hyper-hyperphosphorylated keratin state (upon stimulation) with subsequent abnormal keratin filament reorganization (indicated by large dots). Disease-causing mutations may also result in abnormal filament reorganization due to a hypophosphorylated state, as would be the case for a K8 S73A-like mutation.
a. K8 (in vivo)

b. HK8 (in vivo)

c. K8 + p38 kinase (in vitro)

d. Mix a + c
Keratin 8 phosphorylation by p38 kinase regulates cellular keratin filament reorganization: modulation by a keratin 1-like disease-causing mutation
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