Keratin 8 Phosphorylation by p38 Kinase Regulates Cellular Keratin Filament Reorganization

MODULATION BY A KERATIN 1-LIKE DISEASE-CAUSING MUTATION*

Received for publication, August 9, 2001, and in revised form, December 27, 2001
Published, JBC Papers in Press, January 11, 2002, DOI 10.1074/jbc.M107623200

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Keratin 8 (K8) serine 73 occurs within a relatively conserved type II keratin motif (80NQSLLSPL) and becomes phosphorylated in cultured cells and organs during mitosis, cell stress, and apoptosis. Here we show that Ser-73 is exclusively phosphorylated in vitro by p38 mitogen-activated protein kinase. In cells, Ser-73 phosphorylation occurs in association with p38 kinase activation and is inhibited by SB203580 but not by PD98059. Transfection of K8 Ser-73 → Ala or K8 Ser-73 → Asp with K18 generates normal-appearing filaments. In contrast, exposure to okadaic acid results in keratin filament destabilization in cells expressing wild-type or Ser-73 → Asp K8, whereas Ser-73 → 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 Leu-160 → Pro (157NQSLLQPL → 157NQSPLQPL) leads to epidermolytic hyperkeratosis, we tested and showed that the analogous K8 Leu-71 → Pro leads to K8 hypophosphorylation by p38 kinase in vitro and in transfected cells, likely due to Ser-70 neo-phosphorylation, in association with significant keratin filament collapse upon cell exposure to okadaic acid. Hence, K8 Ser-73 is a physiologic phosphorylation site for p38 kinase, and its phosphorylation plays an important role in keratin filament reorganization. The Ser-73 → Ala-associated filament reorganization defect is rescued by a Ser-73 → Asp mutation. Also, disease-causing keratin mutations can modulate keratin phosphorylation and organization, which may affect disease pathogenesis.

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 to K20) of cytoplasmic proteins that are divided into relatively acidic type I (K9 to K20, pl < 6) and relatively basic type II (K1 to K8, pl ≥ 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, whereas 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 α-helix domain termed the “rod” that is flanked by non-α-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 distribution correlation and other accumulating data (7–11) strongly suggest that phosphorylation plays an important role in regulating the tissue-specific functional roles of the large keratin family.

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). Although 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, which may modulate keratin phosphorylation, have been described. For example, mutations have been described that either introduce a new potential phosphorylation site (e.g. K1 157NQSLLQP → 157NQSPLQPL which renders Ser-159 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 Ser-33 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 (Ser-35→Ala) altered keratin filament organization in transiently transfected cells (27). In addition, transgenic mouse studies showed that K18 Ser-52 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: Ser-23, Ser-431, and Ser-73. Ser-23 is a highly conserved site among all type II keratins, which suggests a common keratin function for this modification, whereas highly conserved site among all type II keratins, which sug-
have been identified: Ser-23, Ser-431, and Ser-73. Ser-23 is a
of stresses in cultured cells and in intact animals (29). In the
in vivo

Although the function of K8 Ser-73 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 pro-
cesses. Here we show that the mitogen-activated protein kinase (MAPK) p38 (reviewed in Refs. 32–35) is a physiologic kinase for K8 Ser-73 phosphorylation, and we demonstrate that K8 Ser-73 phosphorylation plays a significant role in keratin fila-
ment reorganization in response to the phosphatase inhibitor okadaic acid. Since K8 Ser-73 is proximal to a human disease mutation site in epidermal K1 (NQSPLQL of K8), we generated the equivalent K1 mutation in K8 (i.e., NQS-
LLQL→NQSPLQL) and showed that it increased K8 phos-
phorylation, as compared with wild-type K8. This skin disease-
cauing mutation also resulted in significant keratin filament collapse in the presence of okadaic acid. Therefore, K8 Ser-73 phosphorylation plays an important role in modulating keratin filament reorganization. In addition, this is the first demon-
stration 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 are as follows: L2A1 mouse monoclonal antibody (mAb) that recognizes human K1 (24); mAb L41 that recognizes K8 Ser(P)-73 (31); mAb 5B3 that recog-
nizes K8 Ser(P)-431 (30); rabbit Ab 8250 that recognizes K18 Ser(P)-33 (26); rabbit Ab 3055 that recognizes K18 Ser(P)-52 (36); mAb M20 (NeoMarkers; Freemont, CA) that recognizes K8; and anti-FLAG anti-
bodies and enhanced chemiluminescence. Two-dimensional chymotryp-
tic phosphopeptide mapping was carried out exactly as described (30, 40); amino acid electrophoresis was carried out (horizontal) dimension and chromato-
ography in the second (vertical) dimension.

The overlay assay was performed as described (41) with minor mod-
ifications. Briefly, total lysate and K8/18 immunoprecipitates from HT-29 cells were analyzed using SDS-PAGE followed by transfer to a

**Biochemical Methods**—Immunoprecipitation was carried out by sol-
ubilizing 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 phenylmethyl-
sulfonyl fluoride, 10 μM pepstatin, 10 μM leupeptin, 25 μg/ml aprotinin, and 1 μg/ml OA) or by solubilizing cells with 1% Nonidet P-40 in buffer A. After pelleting (15 min; 16,000 × g), keratins were immunoprecipi-
ted from the supernatant using Sepharose-conjugated L2A1 followed

**Cell Transfection and cDNA Constructs**—The K8 mutants K8 Ser-73→Ala (S73A), S73D, and L71P were generated using a Transformer™
mutagenesis kit (CLONTECH Laboratories Inc., Palo Alto, CA) as

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by the supplier. To activate p38 kinase, cells were incubated with An

10 μCi of [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

In Vivo and in Vitro 32P Labeling—In vitro kinase reactions were carried out using K8/18 immunoprecipitates. For each of the keratins 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) and then incubated with 5 μCi of [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 s and then analysis by SDS-PAGE and autoradiography. Metabolic labeling with [32P]orthophosphate was done by incubating cells (in PBS with 0.6% Tween and 0.1% BSA for 2 h (22°C). After washing, the membrane was incubated with anti-p38 antibody for immuno blotting.
**Fig. 1. In vitro K8 phosphorylation by p38 or p42 kinases and mAb LJ4 reactivity with K8 Ser-73 mutants.** A, K5/18 immunoprecipitates from HT-29 cells were prepared using anti-K18 mAb L2A1 or anti-K8 Ser(P)-73 mAb LJ4, followed by SDS-PAGE, and then staining with Coomassie Blue. Note that mAb L41 preferentially recognizes the K8 Ser-73-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). B, H9 cells were co-transfected with WT K18 and WT K5 or with WT K18 and the indicated K8 phosphorylation mutants. K5/18 were precipitated with mAb L2A1 and then analyzed by SDS-PAGE and Coomassie staining. Duplicate K5/18 immunoprecipitates were also separated by SDS-PAGE and 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 K5 (see text). C, in vitro kinase assays were performed using K8/18 immunoprecipitates that were obtained from HT-29 cells. Precipitates were incubated with 5 μCi of [γ-32P]ATP, 20 μM ATP, and 1 unit of p38 or p42 kinase for 15 min followed by quenching with sample buffer then SDS-PAGE analysis, Coomassie staining, and autoradiography (autorad). i.p., immunoprecipitation.

**RESULTS**

Examination of K8 Ser-73 Phosphorylation by Mutational Analysis and by in Vitro Phosphorylation—We identified previously (31) K8 Ser-73 as a K8 phosphorylation site using what we termed a “reverse immunologic” approach. This was aided by an antibody termed LJ4, which was generated by immunizing mice with keratins that were purified from okadaic acid-treated HT-29 cells. As shown previously (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 HT-29 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 Ser-73 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 with the strongly staining K8 S73D species (Fig. 1B).

We compared the in vitro phosphorylation of K8 by the proline-directed MAPKs p38 and p42, given the sequence context of K8 Ser-73 (T1LSSLPL) and the previous observation (31) that K8 Ser-73 becomes phosphorylated during heat stress and apoptosis. As shown in Fig. 1C, p38 kinase generates the radiolabeled HK8 species exclusively (a signature of Ser-73-phosphorylated HK8 species), whereas p42 kinase generates phosphorylated K8 and HK8 (K8 > HK8; compare lanes 2 and 3). Mutation of the two major K8 phosphorylation sites, Ser-23 and Ser-431 (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 Ser-73 abolished formation of the HK8 species and resulted in barely detectable K8 phosphorylation (Fig. 2A, lane 3) that is likely due to Ser-431 phosphorylation (the only other K8 potential proline-directed kinase site, with the sequence D495LTSPG). The specificity of p38 kinase toward K8 Ser-73 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 Ser-23, which is a major basally phosphorylated K8 site (30), is not phosphorylated in vitro by any of the three tested MAPKs, whereas K8 Ser-431 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 Ser-431 and Ser-73 relatively promiscuously, albeit to varied levels, in marked contrast to the selectivity of p38 kinase to the K8 Ser-73 site. In addition, phosphorylation of K8 Ser-73 does not appear to impact on K8 Ser-431 phosphorylation and vice versa.

Evidence of in Vivo K8 Ser-73 Phosphorylation by a p38-like Kinase—Given the findings in Figs. 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 HT-29 cells by An (42), as determined by p38 phosphorylation, is associated with rapid K8 Ser-73 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 Ser-73 phosphorylation (Fig. 3C). In contrast, inhibition of ERK1/2 kinase activation with compound PD98059 did not significantly affect K8 Ser-73 phosphorylation but did inhibit K8 Ser-431 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 unnumbered arrow (Fig. 4, 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 Ser-73.

p38 Kinase Associates with K8/18 and Phosphorylates K8 Ser-73 in Vivo and Binds to K8 in Vitro—We further substantiated in vivo p38 phosphorylation of K8 Ser-73 by comparing K8 Ser-73 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-
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 and then used in an in vitro phosphorylation assay with the indicated kinases. Precipitates were analyzed by SDS-PAGE, Coomassie staining, and then autoradiography.

Fig. 3. Modulation of K8 Ser-73 phosphorylation by activation or inhibition of p38 kinase. A, HT-29 cells were treated with 0.1% MeSO (0-h time point) or with 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, and then blotted with anti-p38 and anti-phospho-p38 kinase antibodies or anti-K8 Ser(P)-73 mAb LJ4. B, HT-29 cells were incubated with MMS and then harvested after the indicated time points, solubilized with 1% Nonidet P-40, followed by immunoprecipitation with mAb L2A1. K8/18 precipitates were separated by SDS-PAGE and then stained with Coomassie Blue or immunoblotted with mAb LJ4. Asterisks in lane 7 represent degraded K8 species. C and D, HT-29 cells were preincubated for 1 h with 20 μM SB203580 (p38 kinase inhibitor) or 100 μM PD98059 (MAPK kinase inhibitor) followed by An treatment for 2 h. K8/18 immunoprecipitates were obtained from 1% Nonidet P-40-solubilized cells and then blotted with anti-K8 Ser(P)-73 (mAb LJ4) or anti-K8 Ser(P)-431 (mAb 5B3). K8 Ser-431 phosphorylation is used as a control since we previously showed that this site becomes phosphorylated upon epidermal growth factor stimulation and that it is likely to be phosphorylated in vivo by p42 MAPK (30).

FLAG and anti-human p38 antibodies. As anticipated, p38 AF is not recognized by phospho-p38 antibody, and K8 Ser-73 phosphorylation increases in BHK cells that overexpress WT but not AF p38 (Fig. 5A, lanes 1–3). In addition, WT and AF p38 kinases co-immunoprecipitate 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., HT-29 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 Ser-73 in vivo.

Effect of Disease-related Keratin Mutations on Keratin Phosphorylation—K8 Ser-73 is part of the sequence 68NQSLLSPL, a sequence that is identical in all type II keratins (except for the Ser-73-equivalent residue which is substituted by Ala in K7,
Note that the vertical dimension and the horizontal dimension) and then chromatography (cellulose plates for two-dimensional separation using electrophoresis - brackets spots 2–5, which are not phosphorylated in K8 in vivo, are phosphorylated in K8 in HK8 and are also generated after in vitro phosphorylation of K8 with p38 kinase. The K8 peptide highlighted by an unnumbered arrow becomes relatively dephosphorylated after MMS treatment in HK8 (compare a with b) and in K8 (not shown).

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. To address this, we focused on one such mutation (Leu-160 → Pro of K1 in a family of patients with epidermolysis bullosa congenita (22)) that occurs in the highly conserved Ser-73-containing domain of K8 (i.e. Leu-71 within GSNQSLSPSPL of K8) by using K8 as a model system (because K1 cDNA is not available). This mutation generates a potential new proline-directed kinase-related site at Ser-70 of K8 (Ser-159 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 9 and 10), which represents the major K18 phosphorylation site (10), thereby indicating specificity of the increased phosphorylation toward the mutant K8. Of note, the L71P K8 mutation inhibits binding of the LJ4 antibody to K8 (Fig. 6B, lane 8) thereby indicating that Leu-71 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 Ser-73 → Ala, Ser-73 → Asp, and Leu-71 → Pro Mutations on Keratin Filament Organization—We tested the effect of the K8 Ser-73 → Ala, Ser-73 → Asp, or Leu-71 → Pro 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...
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K8 Ser-73 Is a Physiologic Substrate for a p38 MAPK—The temporal associations of K8 Ser-73 phosphorylation, as determined by the signature formation of the HK8 species and by reactivity with mAb L14, 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 Ser-73 exclusively, based on HK8 formation (Fig. 1C and Fig. 2A), whereas JNK and p42 kinase resulted in preferential phosphorylation of K8 (due to K8 Ser-431 phosphorylation) with some phosphorylation of the K18 Ser-52 site (Fig. 1B and Fig. 2B). Hence, our data strongly implicate K8 Ser-73 as a physiologic substrate for p38 kinase in vivo, with in vitro phosphorylation (Fig. 4); (iii) inhibition of K8 Ser-73 phosphorylation by the selective p38 kinase inhibitor SB203580 but not by PD98059 (Fig. 3, C 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 Ser-73 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 implicate K8 Ser-73 as a physiologic substrate for p38 kinase and adds K8 to the few known likely physiologic substrates of p38 kinase that include MAPK-activated protein kinase-2 and ribosomal S6 kinase-B (45).

Our assignment of K8 Ser-73 as a physiologic substrate of a p38 kinase pertains in particular to p38a, 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 specity...
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**Fig. 8. Proposed model for the significance of K8 Ser-73 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 Ser-73-independent due to the absence of any detectable basal K8 Ser-73 phosphorylation. Stimulation of cells, as may occur during cell stress or apoptosis, results in K8 Ser-73 phosphorylation via p38 kinase and in “normal” keratin filament reorganization (with increased keratin solubility), which becomes limited upon a K8 Ser-73 → Ala mutation. A K8 Ser-73 → Asp mutation rescues the filament reorganization defect that is caused by blocking Ser-73 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.

Disease-causing Keratins 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 (L160P) occurs at the highly conserved K8 Ser-73-like motif (K8 Leu-71 in SNSPL. 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 Ser-73 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 Ser-73 Phosphorylation Plays an Essential Role in Keratin Filament Organization—One unique feature of the K8 Ser-73 phosphorylation site, as contrasted with other known K8 and K18 phosphorylation sites, is its near-absolute on/off property whereas 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 roles 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 Ser-73 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 Ser-73 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 Ser(P)-73 biologically by rescuing the K8 S73A phenotype (Fig. 7) and biochemically by altering the migration pattern in SDS-PAGE gels from K8 to HK5-like (Fig. 1B).

Another unique feature of the K8 Ser(P)-73 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, Nonidet P-40, Emp, and then post-Emp solubilized fractions (10, 25). Interestingly, the HK8 species are distributed nearly uniformly.
throughout these fractions, whereas keratins that are phosphorylated on K18 Ser-52 or K18 Ser-33 are preferentially found in the cytosolic and Nonidet P-40-containing fractions (10, 26). This implies that keratin species that are typically cytoskeletal and insoluble (K8 Ser-73 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 Ser(P)-73 state and distribution within the different cellular compartments in order to facilitate filament reorganization (Fig. 8).

Acknowledgments—We are indebted to Dr. John Lee for supplying SB203580 and to Kris Morrow for preparing the figures.

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doi: 10.1074/jbc.M107623200 originally published online January 11, 2002

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