Phosphorylation of Human Keratin 8 in Vivo at Conserved Head Domain Serine 23 and at Epidermal Growth Factor-stimulated Tail Domain Serine 431*

(Received for publication, August 12, 1996, and in revised form, January 10, 1997)

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Dynamic phosphorylation is one mechanism that regulates the more than 20 keratin type I and II intermediate filament proteins in epithelial cells. The major type II keratin in “simple type” glandular epithelia is keratin 8 (K8). We used biochemical and mutational approaches to localize two major in vivo phosphorylation sites of human K8 to the head (Ser-23) and tail (Ser-431) domains. Since Ser-23 of K8 is highly conserved among all type II keratins, we also examined if the corresponding Ser-59 in stratified epithelial keratin 6e is phosphorylated. Mutation of K6e Ser-59 abolished its phosphorylation in 32PO4-labeled baby hamster kidney cell transfectants. With regard to K8 phosphorylation at Ser-431, it increases dramatically upon stimulation of cells with epidermal growth factor (EGF) or after mitotic arrest and is the major K8 phosphorylated residue after incubating K8 immunoprecipitates with mitogen-activated protein or cdc2 kinases. A monoclonal antibody that specifically recognizes phosphoserine 431-K8 manifests increased reactivity with K8 and recognizes reorganization of K8/18 filaments after EGF stimulation. Our results suggest that in vivo serine phosphorylation of K8 and K6e within the conserved head domain motif is likely to reflect a conserved phosphorylation site of most if not all type II keratins. Furthermore, K8 Ser-431 phosphorylation occurs after EGF stimulation and during mitotic arrest and is likely to be mediated by mitogen-activated protein and cdc2 kinases, respectively.

The keratin intermediate filament (IF) proteins make up a large family that is represented by more than 20 unique gene products (catalogued as K1–K20) which are specifically expressed in epithelial tissues (for review, see Refs. 1–3). Among cytoplasmic IF proteins, keratins and neurofilaments are unique in that they exist in cells as obligate heteropolymers. All epithelial cells express at least one type I (i.e. one or more of K9–K20) and one type II (i.e. one or more of K1–K8) keratins as obligate noncovalent, epithelial tissue-specific, heteropolymers that coalesce to form typical 10-nm filaments. For example, glandular and secretory epithelia (also called simple type) express K8/18 as their major keratins with variable levels of K19 and K20 (1, 4–8). Similar to all other IF proteins, keratins have the characteristic structural feature of a central 310–350-amino acid coiled coil α-helical domain that is flanked by NH2- and COOH-terminal globular head and tail domains, respectively (for review, see Refs. 3 and 9). As a group, IF proteins are considered relatively insoluble, and this is particularly so for keratins. However, in the case of K8/18, ~5% of the K8/18 pool in asynchronously growing human cultured HT29 colonic epithelial cells consists of soluble tetramers (10).

Although the function of cytoplasmic IF proteins, including keratins, remains poorly understood, their importance in human disease is accumulating at a rapid pace. Mutations in 11 of the 20 keratins (K5/14, K1/10, K4/13, K2/9, K6/16, K17) have already been identified as the cause of several tissue-specific human diseases (for review, see Refs. 11–14). In addition, a mutation in K18 was described recently in a patient with cryptogenic cirrhosis (68). One approach that we have taken toward understanding the function of K8/18 is a detailed characterization of their post-translational modifications to understand their regulation and to provide a handle on their function. The post-translational modifications of K8/18 which have been studied are glycosylation (15, 16) and phosphorylation (17–22; for review, see Ref. 23). These modifications occur in the NH2- and COOH-terminal globular regions of the keratins (23) (termed head and tail domains, respectively, for all IF proteins), which are the regions that impart most of the structural heterogeneity to IF proteins (1–3). With regard to K8/18 phosphorylation, it occurs on serine residues (17) and increases in association with mitosis (18, 22, 24) or under a variety of stress conditions (25, 26). The majority of K8/18 molecules that are phosphorylated are not glycosylated, and vice versa, which suggests that each modification plays a separate role (27). Examples of emerging functions of human K8/18 phosphorylation (for review, see Ref. 23) include: (i) filament reorganization as determined by identification then mutation of a major and highly dynamic physiologic phosphorylation site of human K18 (21); (ii) increasing K8/18 solubility in vitro (19, 25) and in vivo (28); (iii) possibly determining the localization of keratins within specific subcellular domains (22); (iv) regulating the association of K8/18 with the 14-3-3 family of proteins during cell cycle progression, and by doing so, further increasing K8/18 solubility (28); and (v) potentially protecting cells during physiologic stress (25, 26).

The goal of this study was to characterize the major in vivo phosphorylation sites of human K8 and to begin addressing their functional significance. Previous studies showed that un-
nder stimulatory conditions (29–31) such as incubation with epidermal growth factor (EGF) or pro-urokinase, phosphorylation of type II K8 increases preferentially compared with type I K18. Biochemical cleavage at tryptic phospho-site indicated that human K8 phosphorylation involves not only the head domain (as found for K18; Ref. 27) but the head and other non-head domain sites (27, 32). In this study, we used manual Edman degradation of \( ^{32}\)PO\(_4\)-labeled then protease-digested K8 peptides to localize biochemically two major phosphorylation sites of human K8. A mutational approach was then used to confirm Ser-23 and Ser-431 as two major K8 in vivo phosphorylation sites within the head and tail domains, respectively. Since Ser-23 is highly conserved in many type II keratins, we used a mutational approach to demonstrate that K6, an epidermal type II keratin, is also phosphorylated at the corresponding serine site. We also tested the in vitro phosphorylation of Ser-431 by MAP and cdc2 kinases and showed that they are likely to play an in vivo role based on the increased phosphorylation of K8 Ser-431 upon EGF stimulation or mitotic arrest. In addition, a monoclonal antibody that specifically recognizes phosphoserine 431 was generated and used to examine EGF-stimulated cells.

MATERIALS AND METHODS

Cells and Reagents—HT29 (human colon), NIH-3T3 (mouse fibroblast), BHK-21 (hamster kidney) cells, and human placental K8 cDNA were obtained from the American Type Culture Collection. Cells were cultured as recommended by the supplier. Monoclonal antibody (mAb) L2A1 was used, which recognizes human K18 in the context of the heteropolymer K8/18 (15). Other reagents used were: phosphoric acid (\(^{32}\)PO\(_4\)), \(\gamma\)-\(^{32}\)P-ATP (DuPont NEN), trypsin and chymotrypsin ( Worthington Biochemical Corp.), MAP and cdc2 kinases (BioLabs, Beverly, MA); Transformer\(^{\text{TM}}\) mutagenesis kit (Clontech, Palo Alto, CA); and LipofectAMINE liposomes and EGF (Life Technologies, Inc.).

Construction of Mutants and Cell Transformations—Site-directed mutagenesis to generate serine to alanine K8 mutants was done using a Transformer\(^{\text{TM}}\) kit. The cDNA for human K8 and K18 was used as templates as described (21). Mutations were confirmed by sequencing following subcloning into the pMRRB101 mammalian expression vector, with a yeast cytomegalovirus promoter-directed expression. The cDNA for human K6 (33) was kindly provided by Dr. Pierre Coulombe (Johns Hopkins University). Both strands of the entire cDNA for human K8 were sequenced, and the sequence was deposited in GenBank (accession no. U76549). Further confirmation of the nucleotides corresponding to Ser-431 within the sequence 431SPGLSY (not present in published human K8 sequences but present in GenBank sequence X98614) was obtained by sequencing the same region of an independent K8 clone kindly provided by Dr. Robert Oshima (Burnham Institute, La Jolla, CA). Transfection was done using LipofectAMINE as recommended by the manufacturer.

Keratin Immunoprecipitation and High Salt Extraction—Transfected or nontransfected cells were labeled with \(^{32}\)PO\(_4\), by incubating in phosphate-free medium for 30 min followed by adding 200 \(\mu\)Ci/ml \(^{32}\)PO\(_4\) (5 h, 37°C). Labeled cells were solubilized (2 h, 4°C) with 1% Empigen B-21A and 25 \(\mu\)Ci of \(\gamma\)-\(^{32}\)P-ATP, 22 \(\mu\)M ATP, and 1 unit of cdc2 or MAP kinase (total reaction volume = 25 \(\mu\)l). After 10 min at 22°C, 25 \(\mu\)l of 2× sample buffer was added followed by boiling for 1 min, then analysis by analytical or preparative SDS-PAGE.

Generation and Characterization of the 5B3 Antibody—Monoclonal antibody 5B3 was generated after immunizing mice with K8/18 which was purified from okadate-acid-treated HT29 cells. The antibody is an IgG1 and recognizes phosphoserine 431 based on several criteria: (i) it binds to phosphorylated K8 but not to nonphosphorylated K8 as determined by immunoblotting of isoelectric focused SDS-PAGE two-dimensional gels of K8/18 immunoprecipitates; (ii) it binds specifically to a peptide that contains the phosphoserine 431/K8 sequence motif but not to other unrelated peptides; and (iii) the data shown in Fig. 5. Immunofluorescence staining, including keratin and nuclear double staining, was done as described (22). Rabbit anti-K8/18 (antibody 8592) was described previously (67).

RESULTS

Selection of a Transfection System for the Analysis of K8 Phosphorylation—We showed previously that serine is the major phosphorylated residue of K8/18 in human HT29 cells (38). In addition, the two major tryptic phosphopeptides that are metabolically labeled with \(^{32}\)PO\(_4\) in HT29 cells (e.g. Fig. 1B, panel a) were also observed as two major K8 tryptic phosphopeptides in normal colon human biopsies labeled ex vivo with \(^{32}\)PO\(_4\) (21). Our approach for identifying the phosphorylation sites in these two major K8 phosphopeptides was to: (i) use manual Edman degradation of phosphopeptides obtained by trypsin or chymotrypsin digestion of K8 isolated from \(^{32}\)PO\(_4\)-labeled HT29 cells; (ii) generate K8 serine mutants at potential phosphoserines, with mutations chosen to match the manual sequencing results and the amino acid sequence of K8; and (iii) test if phosphorylation at a given tryptic or chymotryptic phosphopeptide is abolished in the mutants.

Toward addressing the above goals, we compared the phosphorylation of K8 in two transfected cell lines (NIH-3T3 and BHK) to phosphorylation of K8 in HT29 cells. As shown in Fig. 1A, K8 in BHK cells manifests a phosphorylation specific activity that is comparable to K8 in HT29 cells. In addition, there is significant overlap of the tryptic (T1, T2) and chymotryptic (C1–C4) phosphopeptides of K8 isolated from HT29 and BHK cells (Fig. 1B). Therefore, we used BHK cells for the transfection and characterization of K8 phosphorylation experiments as described below (K8 expressed in NIH-3T3 cells manifested a peptide map pattern similar to that of K8 isolated from HT29 cells; not shown).

Identification of Ser-23 and Ser-431 as Two Major Human K8 Phosphorylation Sites—We sequenced the cDNA for human K8 since assignment of specific peptides and potential serines depends on having a precise localization of Arg/Lys (for tryptic fragments), Phe/Tyr/Trp (for chymotrypsin fragments), and serines within the proteolytic fragments. The human K8 protein contains 61 potential serine phosphorylation sites (not shown). Minor differences were noted between our sequence (see "Materials and Methods") and two other available sequences, but one relevant difference was the presence of a serine (Ser-431) within the K8 peptide 426YGGLTSPG in contrast to the reported K8 sequences 430YGGDLTDPG (39) and 426YGGQSGAG (40). An identical 426YGGLTSPG sequence is

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found in mouse (41) and rat K8 (42) and in an independent human K8 cDNA clone (not shown); and notably, this sequence contains a serine that is in the fifth position after cleavage of Tyr-426 by chymotrypsin (see below).

Each of the $^{32}$PO$_4$-labeled peptides T1, T2, C1–C4 shown in Fig. 1B (panels c and f) were isolated following by manual Edman degradation. As shown in Table IA, three of the peptides (T2, C1, and C4) afforded informative degradation with release of the $^{32}$PO$_4$-serine at specific cycles, whereas a significant portion of the counts of the other three peptides (T1, C2, C3) remained within the filter and were noninformative. In the case of peptide T2 Edman degradation, analysis of the K8 sequence indicates that Ser-1, 8, 23, 133, 252, and 273 are potential phosphorylation sites since they immediately follow an adjacent Arg/Lys. To determine the relationship between T2 and C1/C4, we tried trypsin digested individually purified C1 and C4 peptides (Table IB), which resulted in release of radioactivity of the C4 peptide at the first cycle. This, coupled with the known K8 sequence, indicates that T2 and C4 are related and that phosphorylation of T2/C4 likely occurs on Ser-23 in 19FSSRSY. Using a similar analysis, the results of Edman degradation of C1 (Table IA) and Edman degradation of trypsin-digested C1 (Table IB) suggest that Ser-290, 423, 431, and/or 441 may be phosphorylated (Table II). Although phosphorylation of Ser-14, 42 and 456 (Table II) may correspond to chymotryptic peptide C1 (i.e. release at fifth cycle), tryptic digestion of C1 followed by Edman degradation (Table II) excludes phosphorylation at those sites. This is due to the presence of trypsin-sensitive Lys/Arg (arrows, bottom three peptides, Table II), which would be expected to change the cycle number of radioactivity release after Edman degradation of peptide C1 from the observed fifth cycle (Table IB) to a first or second cycle (Table II). The reason for the resistance of T1, C2, C3, or chymotrypsin-digested T1 peptides to Edman degradation (Table I) is unclear, but it may

### Table I

| Peptide | Initial cpm | Peak cycle | cpm range of other cycles | Remaining filter cpm | Total cycles |
|---------|-------------|------------|--------------------------|----------------------|-------------|
| T1      | 4,500       | no peak    | 41–68                    | 2,229                | 29          |
| T2      | 15,000      | 1st (7531) | 43–151                   | 184                  | 14          |
| C1      | 3,000       | 5th (415)  | 51–86                    | 186                  | 8           |
| C2      | 4,000       | no peak    | 34–193                   | 1,780                | 15          |
| C3      | 2,500       | no peak    | 38–65                    | 515                  | 15          |
| C4      | 7,500       | 4th (1019) | 77–285                   | 485                  | 8           |

### Table II

| Peptide | Initial cpm | Redigested with | Peak cycle | cpm range of other cycles | Remaining filter cpm | Total cycles |
|---------|-------------|-----------------|------------|--------------------------|----------------------|-------------|
| T1      | 2,700       | chymotrypsin    | no peak    | 41–60                    | 1,272                | 29          |
| C1      | 2,000       | trypsin         | 5th (840)  | 56–100                   | 297                  | 27          |
| C4      | 2,400       | trypsin         | 1st (640)  | 52–69                    | 80                   | 80          |
reflect cyclization with inaccessibility of the peptide and/or phosphorylation at a distant serine residue that is inefficiently cleaved after more than 15 Edman degradation cycles (43). Given the above biochemical analysis, we generated several serine mutant human K8 constructs to confirm the phosphorylation sites in peptides C4 (or the related T2) and C1. This was done by cotransfection of wild type K18 with mutant or wild type K8 into BHK cells, followed by metabolic labeling with $^{32}$PO$_4$ and chymotryptic peptide mapping of isolated K8. All of the mutants resulted in a “wild type” pattern (Table II) similar to that in Fig. 1, except for the Ser-23 mutant, which showed an absence of $^{32}$PO$_4$-labeled peptides C4 (Fig. 2B, panels a and b), and T2 (Table II, peptide map not shown), and the Ser-431 mutant, which showed an absence of $^{32}$PO$_4$-labeled peptide C1 (Fig. 2B, panels c and d). In addition, the S23A/S431A double mutant showed absence of both peptides C1 and C4 as expected (Fig. 2B, panels e and f). Only a partial decrease in the overall phosphorylation of the double mutant was noted (Fig. 2A) secondary to the presence of several other K8 peptides that are radiolabeled in BHK cells.

Ser-23 of K8 is a Common Phosphorylation Site among Type II Keratins—Analysis of head domain sequences of type II keratins (K1–K8) shows a striking degree of conservation of Ser-23 among all type II keratins (Table III, shaded residues). This led us to test if phosphorylation of this serine is conserved in other type II keratins. For this, we tested whether the equivalent serine in K6 (i.e., Ser-59) is phosphorylated, by comparing the tryptic peptide maps of wild type and Ser-59 Ala (S59A) K6 after transfection into BHK cells and then labeling with $^{32}$PO$_4$. As shown in Fig. 3A, high levels of K6/18 can be coexpressed in transfected BHK cells, and tryptic peptide mapping analysis indicates that the Ser-59 of K6 is phosphorylated in vivo in BHK cells (Fig. 3, b–d). Mutation of Ser-59 of K6e resulted in the absence of a labeled phosphopeptide (Fig. 3, panel c), which was present in wild type K6 (Fig. 3, panel b) and in the mix map (Fig. 3, panel d; peptide indicated by an arrow). Similar results were obtained, in terms of the disappearance of a labeled phosphopeptide for the S59A K6e mutant, after chymotryptic peptide mapping (not shown). Although phosphorylation of the equivalent serine residue in other type II keratins remains to be confirmed, its observed phosphorylation in K6 and K8 and the conservation of its motif suggest that other type II keratins are likely to be phosphorylated at the corresponding site.

Ser-431 of K8 Is Phosphorylated in Vitro by MAP and cdc2 Kinases and in Vivo upon EGF Stimulation and Mitotic Arrest—Ser-431 is proximal to proline within the sequence 429LTSPGL, which suggests that one or more proline-directed kinases, such as MAP and cdc2 kinases, is/are likely to phosphorylate this residue. Phosphorylation of K8 Ser-431 by MAP kinase (Fig. 4D) or cdc2 kinases and in vivo upon EGF stimulation and mitotic arrest (Fig. 4C) can be tested by cotransfection of wild type or Ser-431 Ala (S431A) K8 constructs with wild type K18 into BHK cells, followed by metabolic labeling with $^{32}$PO$_4$ and chymotryptic peptide mapping, as described in the Fig. 2 legend, to generate the T1 and T2 peptides (trypsin digestion) or C1–C4 peptides (chymotryptic digestion). + and − correspond to the presence or absence, respectively, of the indicated phosphopeptide. N.A., not applicable. Serine numbers highlighted in bold indicate the confirmed K8 phosphorylation sites as determined by the disappearance of a tryptic and/or chymotryptic peptide.

### Table II

| Domain | Potential phosphopeptide | Mutant ser | T1 | T2 | C1 | C2 | C3 | C4 |
|--------|--------------------------|------------|----|----|----|----|----|----|
| Head   | 19F · ·  S S R↓ S*        | 23         | +  | -  | +  | +  | +  | -  |
| Rod    | 285Y · ·  E E L Q S*     | 290        | N.A | N.A | +  | +  | +  | +  |
| Tail   | 418Y · ·  A G G L S*     | 423        | N.A | N.A | +  | +  | +  | +  |
| Tail   | 426Y · ·  G G L T S*     | 431        | N.A | N.A | -  | +  | +  | +  |
| Tail   | 436Y · ·  S L G S S*     | 441        | N.A | N.A | +  | +  | +  | +  |
| Head   | 9Y · ·  K↓ V S T S*      | 14         | Not tested |
| Head   | 37F · ·  S R↓ V G S*     | 42         | Not tested  |
| Tail   | 451F · ·  S R↓ T S S*    | 456        | Not tested  |
cdc2 (not shown, but pattern identical to that of MAP kinase shown in Fig. 4D) is abolished if K8 S431A is used as a substrate (in the context of a K8/18 immunoprecipitate). Second, in vitro phosphorylation of wild type K8 by MAP and cdc2 kinases (Fig. 4E, panels a–d) results in selective phosphorylation of the Ser-431-containing K8 peptide.

To assess the physiologic significance of in vitro phosphorylation of Ser-431 by MAP and cdc2 kinases, we compared the chymotryptic phosphopeptide maps of basally phosphorylated K8 in HT29 cells that are asynchronously growing (not shown; but similar to Fig. 1B, panel d), serum-starved (Fig. 4E, panel b), serum-starved then EGF stimulated (Fig. 4E, panel e) or arrested at G2/M (Fig. 4E, panel f). The basal low level phosphorylation of Ser-431 increased significantly after EGF stimulation and during mitotic arrest (Fig. 4E, compare panels e and f with b) in association with the overall increase in K8 phosphorylation upon EGF stimulation (Fig. 4B) or mitotic arrest (Fig. 4C).

Phosphorylation of Ser-431 was also assessed using mAb 5B3, which was generated after immunizing mice with hyperphosphorylated keratins. This antibody specifically recognizes the phosphoserine 431-containing motif (see “Materials and Methods”), and its reactivity is abolished upon mutation of K8 Ser-431 (Fig. 5A). Given the results shown in Fig. 4, B and E, we examined K8/18 immunoprecipitates (obtained using mAb L2A1, which recognizes phosphorylated and nonphosphorylated K8/18) by immunoblotting using mAb 5B3. As shown in Fig. 5B, EGF stimulation of HT29 cells results in increased binding of mAb 5B3 as would be expected based on Ser-431 phosphorylation. Immunofluorescence staining of HT29 cells with mAb 5B3, before and after EGF treatment, showed reorganization of the keratin filaments that are recognized by the antibody (Fig. 5C, compare panels a and b). This reorganization was manifested by a ring-like cortical enhancement of filament staining near the cell periphery. This filament reorganization is not related to a mitotic event as determined by nuclear double staining (Fig. 5C) and appears to overlap with the overall keratin staining that was visualized using rabbit anti-
The major findings of this study are as follows. (i) Two major in vivo phosphorylation sites were identified in human K8, one in the head domain (Ser-23) and one in the tail domain (Ser-431); (ii) The K8 head domain phosphorylation site, which is highly conserved among all type II keratins, is also phosphorylated in K6e. Hence, this conserved head domain serine is likely to be a common phosphorylation site among all type II keratins. (iii) Phosphorylation of K8 tail-domain Ser-431 is likely to involve MAP and cdc2 kinases during growth factor stimulation and mitosis, respectively, based on in vitro and in vivo evidence.

**DISCUSSION**

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**General Features of K8 Phosphorylation**—In characterizing K8 phosphorylation, we utilized a combined approach of manual Edman degradation of in vivo 32PO4-labeled K8 and then protease-generated peptides, sequence information, and a molecular approach to confirm biochemically suggested phosphorylation sites. The protease utilized was important in that one of two major trypsin-generated phosphopeptides consists of at least three species (i.e., C1–C3) after chymotrypsin digestion (Tables I and II). The generation of C1–C3 peptides appears possible only upon chymotrypsin digestion of the intact K8 protein (e.g., Table IA) but not after chymotrypsin digestion of tryptic peptide T1 (Table IB). Manual Edman degradation was not effective in releasing the phosphoamino acid for C2 and C3 peptides and did not aid in identifying these two phosphorylation sites. The in vitro phosphorylation of K8 by MAP and cdc2 kinases exclusively at the C1 peptide indicate that C2 and C3 are more likely to be unique phosphorylation sites rather than partially digested peptides that are related to C1.

The kinase(s) that phosphorylates Ser-23 of K8 is(are) not known, and the amino acid sequence context of phosphoserine 23 does not provide a clear structural motif for a specific kinase. Several in vitro phosphorylation sites were recently identified biochemically after phosphorylating purified K8 with cAMP-dependent protein kinase (protein kinase A) (57). One of the sites corresponds to Ser-23, which raises the possibility that protein kinase A may be a relevant physiologic kinase for Ser-23 of K8. To that end, phosphorylation of K8/18 increased in cultured human intestinal Caco-2 cells upon treatment with forskolin (58). However, activation of protein kinase A by incubating IIT29 cells with 8-bromo-cAMP or forskolin did not increase K8 phosphorylation (38), and incubation of rat hepatocytes (59) or dog thyroid follicle cells (60) with dibutyryl-cAMP did not increase keratin phosphorylation. In addition, phosphorylation of purified rat K8 by protein kinase A results in the phosphorylation of several sites (19, 57) so that the pertinent kinase(s) involved in Ser-23 phosphorylation remains to be investigated. In the case of Ser-431 phosphorylation, both cdc2 and MAP kinases appear to be relevant physiologic kinases as supported by the consensus sequence of Ser-431, by the specific enhancement of Ser-431 phosphorylation upon EGF stimulation or mitotic arrest, and the phosphorylation by these kinases exclusively on Ser-431.

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**Table III**

The serine 23-containing motif of K8 is conserved among type II keratin head domains

| Type II keratin | Sequence | Reference |
|----------------|----------|-----------|
| H - K8         | R A F S S R Y T | Krauss and Franke, 1990; Yamamoto et al., 1990 |
| X - K8         | G G F S | Franz and Franke, 1986 |
| M, R - K8      | F S | Morita et al., 1988 (M); Hsieh et al., 1992 (R) |
| H - K1         | G G G F S | Johnson et al., 1985 |
| H - K2e        | G G G F S | Collin et al., 1992a |
| H - K2p        | S S G F S | Collin et al., 1992b |
| H - K3         | G G G F S | Klinge et al., 1987 |
| H - K4         | G G G F S | Knapp et al., 1986 |
| H - K5         | Y G F S | Lersch et al., 1989 |
| H - K6a-f      | G G G F S | Tyner and Fuchs, 1986; Takahashi et al., 1995 |
| H - K7         | P V T A | Glass and Fuchs, 1988 |

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**FIG. 3.** Phosphopeptide mapping of wild type (WT) and Ser-59 → Ala human K6e. Panel A, wild type or a Ser-59 → Ala (S59A) human K6e was cotransfected with human K18 into BHK cells. After 3 days, the keratin fraction was isolated by high salt extraction as described under “Materials and Methods” followed by SDS-PAGE analysis. Panels b–d, BHK cells, cotransfected with wild type K18 + K6 or wild type K18 + S59A K6, were labeled with 32PO4 (5 h, 250 μCi/ml) followed by high salt extraction and then isolation of K6 from preparative gels and tryptic peptide mapping. The mixed peptide map (panel d) shows equal counts of wild type K6 and the S59A K6 that were spotted. The arrow in panel d highlights the S59A-containing phosphopeptide that is not seen upon mutation of Ser-59 to Ala (panel c).
Phosphorylation of K8 at Ser-23 and Ser-431 indicates that both the head and tail domains of K8 are phosphorylated as shown previously for K1 (61), another type II keratin. In the case of type I keratins, the only studied example is K18, which appears to have its phosphorylation preferentially in the head domain (21, 27). The phosphorylation of type I and type II keratins appears to be independent in that mutation of a major phosphorylation site of K18 does not affect K8 phosphorylation (21), and mutation of Ser-23/431 K8 does not affect overall K18 phosphorylation or the tryptic phosphopeptide pattern of K18 phosphorylation (not shown).

Functional Relevance of Ser-23 and Ser-431 Phosphorylation of K8—Both Ser-23 of K8 and Ser-59 of K6e are contained within the highly conserved motif SRSX underlined serine is phosphorylated, and X represents an aliphatic/aromatic residue), which is found within all type II keratins and is conserved across species except for Xenopus (Table III). The in vitro phosphorylation of the serine within this motif in K8 and K6e suggests that the equivalent serines in other type II keratin phosphorylation or the tryptic phosphopeptide pattern of K8 phosphorylation (not shown).

Panel A, K8/18 immunoprecipitates were prepared from detergent-solubilized HT29 cells followed by heating (90 °C, 1 min) to destroy any associated kinase activity. In vitro phosphorylation using 1 unit of MAP orcdc2kinases was then carried out as described under “Materials and Methods.” Phosphorylation of an identical immunoprecipitate, in the absence of an added kinase, provided a blank signal after an exposure time that was 10 × that of the kinase containing samples (not shown). Panel B, HT29 cells were cultured in the absence of serum for 14 h followed by labeling in phosphate-free, serum-free medium for 5 h with the addition of EGF (1 μg/ml) for the last h of labeling. Cells were then solubilized followed by immunoprecipititation of K8/18 and then SDS-PAGE analysis. Panel C, asynchronously growing G0/G1- or G2/M-arrested HT29 cells were labeled with 32PPO4 for 5 h followed by immunoprecipitation of K8/18. Panel D, BHK cells were cotransfected with wild type (WT) K18 and one of three K8 constructs (wild type, S431A, or S23A). After 3 days, K8/18 immunoprecipitates were prepared in duplicate, followed by heating (90 °C, 1 min) and then in vitro phosphorylation using MAP kinase of one set of the duplicates as in panel A. In vitro phosphorylation withcdc2kinase using the second duplicate samples gave similar results (not shown). Panel E, 32PPO4-labeled K8 was isolated using preparative gels from the corresponding immunoprecipitates shown in panels A–C, followed by chymotryptic peptide mapping as described under “Materials and Methods.” For each map, 10,000 cpm were spotted (5,000 cpm from samples a and b, each, for panel c). The map of b + d was similar to that shown in c (not shown). Arrows indicate the Ser-431-containing peptide.

Phosphorylation of K8 at Ser-23 and Ser-431 indicates that both the head and tail domains of K8 are phosphorylated as shown previously for K1 (61), another type II keratin. In the case of type I keratins, the only studied example is K18, which appears to have its phosphorylation preferentially in the head domain (21, 27). The phosphorylation of type I and type II keratins appears to be independent in that mutation of a major phosphorylation site of K18 does not affect K8 phosphorylation (21), and mutation of Ser-23/431 K8 does not affect overall K18 phosphorylation or the tryptic phosphopeptide pattern of K8 phosphorylation (not shown).

Functional Relevance of Ser-23 and Ser-431 Phosphorylation of K8—Both Ser-23 of K8 and Ser-59 of K6e are contained within the highly conserved motif SRSX underlined serine is phosphorylated, and X represents an aliphatic/aromatic residue), which is found within all type II keratins and is conserved across species except for Xenopus (Table III). The in vitro phosphorylation of the serine within this motif in K8 and K6e suggests that the equivalent serines in other type II keratin phosphorylation or the tryptic phosphopeptide pattern of K8 phosphorylation (not shown).

Panel A, K8/18 immunoprecipitates were prepared from detergent-solubilized HT29 cells followed by heating (90 °C, 1 min) to destroy any associated kinase activity. In vitro phosphorylation using 1 unit of MAP or cdc2 kinases was then carried out as described under “Materials and Methods.” Phosphorylation of an identical immunoprecipitate, in the absence of an added kinase, provided a blank signal after an exposure time that was 10 × that of the kinase containing samples (not shown). Panel B, HT29 cells were cultured in the absence of serum for 14 h followed by labeling in phosphate-free, serum-free medium for 5 h with the addition of EGF (1 μg/ml) for the last h of labeling. Cells were then solubilized followed by immunoprecipitation of K8/18 and then SDS-PAGE analysis. Panel C, asynchronously growing G0/G1- or G2/M-arrested HT29 cells were labeled with 32PPO4 for 5 h followed by immunoprecipitation of K8/18. Panel D, BHK cells were cotransfected with wild type (WT) K18 and one of three K8 constructs (wild type, S431A, or S23A). After 3 days, K8/18 immunoprecipitates were prepared in duplicate, followed by heating (90 °C, 1 min) and then in vitro phosphorylation using MAP kinase of one set of the duplicates as in panel A. In vitro phosphorylation with cdc2 kinase using the second duplicate samples gave similar results (not shown). Panel E, 32PPO4-labeled K8 was isolated using preparative gels from the corresponding immunoprecipitates shown in panels A–C, followed by chymotryptic peptide mapping as described under “Materials and Methods.” For each map, 10,000 cpm were spotted (5,000 cpm from samples a and b, each, for panel c). The map of b + d was similar to that shown in c (not shown). Arrows indicate the Ser-431-containing peptide.

Desmoplakin. For example, despite colocalization of keratins with desmoplakin (63) in a manner that is regulated negatively by phosphorylation of a COOH-terminal site of desmoplakin (64), desmoplakin did not bind to K8 using an in vitro assay under conditions that other type II keratins bound (62). In exploring this question, desmoplakin does not coimmunoprecipitate with K8/18 after solubilizing cells with nonionic detergents (most of desmoplakin remains insoluble under these conditions in HT29 cells) or with the zwitterionic detergent Empigen (not shown), which solubilizes a significant component of the cytoskeletal/filamentous K8/18 (28, 34). However, this does not exclude a potential weak interaction between desmoplakin and K8 which becomes abolished after Empigen solubilization.

With regard to Ser-431, its phosphorylation increases upon stimulation of cells with EGF (Fig. 4), which suggests a role in mitogen-induced signaling. This site is conserved in K8 sequences of mouse (41) and rat (42), but not Xenopus (44). The 429LTSPG sequence of K8 is also not conserved in the tail domain of other type II keratins, which suggests a specific function for this site which is related to glandular epithelia. The significance of Ser-431 phosphorylation after EGF exposure to cells and its importance in regulating downstream events of EGF-receptor or other mitogen-induced activation remain to be determined. The known activation of MAP kinase by EGF and the exclusive phosphorylation of Ser-431 in vitro...
Phosphorylation of Ser-431 also increased upon mitotic arrest (Fig. 4), but the biologic significance of this phosphorylation in terms of progression through mitosis or other mitosis-related events remains to be determined. Approaches such as the generation of antibodies that are unique to specific phosphopeptides (for review, see Refs. 65 and 66) should provide additional functional insights into K8/18 phosphorylation and the relationship and sequence of events of the multiple K8 and K18 phosphorylation sites.

**Acknowledgment**—We are very grateful to Dr. Pierre Coulombe for providing the K8e cDNA, Dr. Werner Franke and his laboratory for sharing DNA sequencing results, Dr. Robert Oshima for providing an independent human K8 cDNA, Kris Morrow for constructing the figure, and Letty S. Esguerra and Romola L. Breckenridge for preparing the manuscript.

**REFERENCES**

1. Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R. (1982) Cell 31, 11–24
2. Coulombe, P. A. (1993) Curr. Opin. Cell Biol. 5, 17–29
3. Fuchs, E., and Weber, K. (1994) Annu. Rev. Biochem. 63, 345–382
4. Moll, R., Schiller, D. L., and Franke, W. W. (1990) J. Cell Biol. 111, 567–580
5. Moll, R., Zimbelman, R., Goldschmidt, M. D., Keith, M., Lauffer, J., Kasper, M., Koch, P. J., and Franke, W. F. (1993) Differentiation 53, 75–93
6. Stasiak, P. C., Pursis, P. E., Leigh, I. M., and Lane, E. B. (1989) J. Invest. Dermatol. 92, 707–716
7. Eckert, R. L. (1988) Proc Natl. Acad. Sci. U. S. A. 85, 1114–1118
8. Caldec, N., and Quaroni, A. (1993) Differentiation 53, 95–104
9. Steinert, P. M., and Roop, D. R. (1993) Ann. Rev. Biochem. 57, 593–625
10. Chou, C.-F., Riopel, C. L., Rott, L. S., and Omary, M. B. (1993) J. Cell Sci. 105, 433–445
11. Fuchs, E., and Coulombe, P. A. (1992) Cell 69, 890–902
12. Steiner, P. M., and Roop, D. R. (1993) Trends Genet. 9, 280–284
13. Chung, T., and Omary, M. B. (1993) Cell 75, 941–955
14. McLean, W. H. I., and Lane, E. B. (1995) Curr. Opin. Cell Biol. 7, 118–125
15. Chou, C.-F., Smith, A. J., and Omary, M. B. (1992) J. Biol. Chem. 267, 1901–1906
16. Ku, N.-O., and Omary, M. B. (1995) J. Biol. Chem. 270, 11820–11827
17. Oshima, R. G. (1982) J. Biol. Chem. 257, 3414–3421
18. Celis, J. E., Larsen, P. M., Fey, S. J., and Celis, A. (1983) J. Cell Biol. 97, 1428–1434
19. Yano, T., Tokui, T., Nishi, Y., Nichizawa, K., Shibata, M., Kikuchi, K., Tsuiki, S., Yamauchi, T., and Inagaki, M. (1991) Eur. J. Biochem. 197, 281–289
20. Omary, M. B., Baxter, W. T., Chou, C.-F., Riopel, C. L., Lin, W. Y., and Strulovici, B. (1996) J. Cell Biol. 135, 583–593
21. Ku, N.-O., and Omary, M. B. (1994) J. Cell Biol. 127, 161–171
22. Liao, J., Lovett, L. A., Ku, N.-O., Fernandez, R., and Omary, M. B. (1995) J. Cell Biol. 131, 1291–1301
23. Ku, N.-O., Liao, J., Chou, C. F., and Omary, M. B. (1996) Cancer Metastasis Rev. 15, 429–444
24. Chou, C.-F., and Omary, M. B. (1994) J. Cell Biol. 120, 357–370
25. Boyle, W. J., Van Der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
26. Dong, D. L.-Y., Xu, Z.-S., Chevrier, M. R., Cotter, R. J., Cleveland, D. W., and Hart, G. W. (1993) J. Biol. Chem. 268, 16679–16687
27. Chou, C.-F., and Omary, M. B. (1995) FEBS Lett. 282, 200–204
28. Yamamoto, R., Kao, L.-C., McKnight, C. E., and Strauss, J. F., III (1990) Mol. Endocrinol. 4, 570–574
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40. Krauss, S., and Franke, W. W. (1990) Gene (Amst.) 86, 241–249
41. Morita, T., Tondella, M. L. C., Takemoto, Y., Hashido, K., Ichinose, Y., Nozaki, M., and Matsuhiro, A. (1988) Gene (Amst.) 68, 159–117
42. Hsieh, J.-T., Zhou, X.-H., Liew, C.-C., and Chung, L. W. K. (1992) J. Biol. Chem. 267, 2303–2310
43. Sullivan, S., and Wong, T. W. (1991) Anal. Biochem. 197, 65–68
44. Franz, J. K., and Franke, W. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6475–6479
45. Johnson, L. D., Idler, W. W., Zhou, X.-M., Roop, D. R., and Steinert, P. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1896–1900
46. Collin, C., Moll, R., Kubicka, S., Ouhayoun, J.-P., and Franke, W. W. (1992a) Exp. Cell Res. 202, 132–141
47. Collin, C., Ouhayoun, J. P., Grund, C., and Franke, W. W. (1992b) Differentiation 51, 137–148
48. Klinge, E. M., Sylvestre, Y. M., Freedberg, I. M., and Blumenberg, M. (1987) J. Mol. Biol. 24, 319–329
49. Knapp, B., Rentrop, M., Schweizer, J., and Winter, H. (1986) Nucleic Acids Res. 14, 751–763
50. Lersch, R., Stellmach, V., Stocks, C., Giudice, G., and Fuchs, E. (1989) Mol. Cell Biol. 9, 3685–3697
51. Tyner, A. L., Eichman, M. J., and Fuchs, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4683–4687
52. Glass, C., and Fuchs, E. (1988) J. Cell Biol. 107, 1337–1350
53. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
54. Chou, Y.-H., Bischoff, J. R., Beach, D., and Goldman, R. D. (1990) Cell 62, 1063–1071
55. Tsujimura, K., Ogasawara, M., Takeuchi, Y., Imajoh-Ohmi, S., Ha, M. H., and Inagaki, M. (1994) J. Biol. Chem. 269, 31097–31106
56. Matsuoka, Y., Nishizawa, K., Yano, T., Shibata, M., Ando, S., Takahashi, T., and Inagaki, M. (1992) EMBO J. 11, 2895–2902
57. Ando, S., Tokui, T., Yano, T., and Inagaki, M. (1996) Biochem. Biophys. Res. Commun. 221, 67–71
58. Baricault, L., de Nècheaud, B., Sapin, C., Codogno, P., Denoulet, P., and Trugnan, G. (1994) J. Cell Sci. 107, 2909–2918
59. Kawahara, H., Cadrin, M., and French, S. W. (1990) Life Sci. 47, 859–863
60. Deery, W. J. (1995) Cell Motil. Cytoskeleton 26, 325–339
61. Steinert, P. M. (1988) J. Biol. Chem. 263, 13333–13339
62. Kuokis, P. D., Hutton, E., and Fuchs, E. (1994) J. Cell Biol. 127, 1049–1060
63. Stappenbeck, T. S., Borrselaeger, E. A., Corcoran, C. M., Luu, H. H., Virata, M. L. A., and Green, K. J. (1993) J. Cell Biol. 123, 691–705
64. Stappenbeck, T. S., Lamb, J. A., Corcoran, C. M., and Green, K. J. (1994) J. Biol. Chem. 269, 29351–29354
65. Inagaki, N., Ito, M., Nakano, T., and Inagaki, M. (1994) Trends Biochem. Sci. 19, 448–452
66. Liao, J., Ku, N.-O., and Omary, M. B. (1996) Electrophoresis 17, 1671–1676
67. Ku, N.-O., Michie, S., Oshima, R. G., and Omary, M. B. (1995) J. Cell Biol. 131, 1303–1314
68. Ku, N.-O., Wright, T. L., Terrault, N. A., Gish, R., and Omary, M. B. (1997) J. Clin. Invest. 99, 19–23