Characterization of the Major Physiologic Phosphorylation Site of Human Keratin 19 and Its Role in Filament Organization*

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Keratin polypeptide 19 (K19) is a type I intermediate filament protein that is expressed in stratified and simple-type epithelia. Little is known regarding K19 regulation or function, and the only other type I keratin that has been studied in terms of regulation is keratin 18 (K18). We characterized K19 phosphorylation as a handle to study its function. In vivo, serine is the major phosphorylated residue, and phosphopeptide mapping of $^{32}$PO$_4$-labeled K19 generates one major phosphopeptide. Edman degradation suggested that the radiolabeled phosphopeptide represents K19 Ser-10 and/or Ser-35 phosphorylation. Mutation of Ser-10 or Ser-35 followed by transfection confirmed that Ser-35 is the major K19 phosphorylation site. Transfection of Ser-35→Ala K19 showed a filament assembly defect as compared with normal or with Ser-10→Ala K19. Comparison of K18 and K19 phosphorylation features in interphase cells showed that both are phosphorylated primarily at a single site, preferentially in the soluble versus the insoluble keratin fractions. K19 has higher basal phosphorylation, whereas K18 phosphorylation is far more sensitive to phosphatase type I and IIA inhibition. Our results demonstrate that Ser-35 is the major K19 interphase phosphorylation site and that it plays a role in keratin filament assembly. K19 and K18 phosphorylations share some features but also have distinct properties that suggest different regulation of type I keratins within the same cells.

The cytoskeleton of most mammalian cells consists of three distinct major protein families: microtubules, microfilaments, and intermediate filaments (1). Among these three cytoskeletal protein groups, intermediate filaments (IF)† have several unique features (2–6). For example, IF proteins make up a complex and diverse family; they are expressed in a tissue-preferential manner; their functions are poorly understood; they have a characteristic structure that consists of a central coiled-coil $\alpha$-helical domain (termed “rod”) that is flanked by globular N-terminal (termed “head”) and C-terminal (termed “tail”) domains; and several mutations have been identified in IF proteins that result in a variety of skin, oral, and ocular diseases. Another feature of IF proteins is their expression in the nucleus (i.e., the lamina) and in the cytoplasm (2–4). Among the cytoplasmic IF proteins, keratins make up the largest family and are expressed specifically in epithelial cells in a cell-specific manner (7, 8). Keratins (K) include more than 20 unique gene products (termed K1–K20) that are divided into type I (K9–K20) and type II (K1–K8) (7, 8). Most epithelial cells express at least one type I and one type II keratin as their predominant IF protein complement in an epithelial cell-specific manner (7). For example, basal keratinocytes express K5/14 with little if any K1/10, whereas suprabasal keratinocytes lose most if not all of their K5/14 and express K1/10. In addition, “simple-type” epithelia as found in the liver, exocrine pancreas, and intestine express K8/18 with various levels of K19 and K20. Among the keratins, K19 is unique in that it has a very short 13-amino acid tail domain, it is expressed in stratified and simple-type epithelia and in hair follicles, and it may serve as a skin stem cell marker (9–13).

Although the likely multidimensional functional roles of keratins are still under active study, two important functions are well established, and keratin phosphorylation appears to play a role in both functions. The first function is maintaining cell mechanical integrity as has been clearly demonstrated from the skin and ocular keratin-associated diseases (reviewed in Refs. 4–6) and from transgenic models that manifest a liver phenotype (reviewed in Ref. 14). In the case of the liver, there is a strong correlation between exposure to a variety of cell stresses, with resultant hepatocyte toxicity, and a significant increase in keratin phosphorylation (reviewed in Refs. 15 and 16). To that end, expression of K18 that is mutated at its major phosphorylation site (serine 52) in transgenic mice is associated with a significant increase in susceptibility to hepatotoxic injury as compared with transgenic mice that overexpress wild type K18 (17). A second functional role for keratins is the interaction with other cellular proteins such as the 14-3-3 protein family, which presumably regulates the known interaction of 14-3-3 proteins with a number of their binding partners (18). The keratin/14-3-3 association is cell-cycle-regulated in that it occurs with K18 (but not K19 or K8) only during the S and G$_2$/M phases of the cell cycle in a reversible fashion (19) upon phosphorylation of K18 serine 33 (18). Therefore, the characterization of keratin phosphorylation and its significance is emerging as a productive approach for understanding not only keratin regulation but also its function.

In this report we focus on characterizing K19 phosphorylation and begin to address its significance. The only keratins that have been studied in detail with respect to their phosphorylation are K1 (20), K8, and K18 (reviewed in Refs. 15 and 16). However, most if not all of the 20 keratins are likely to be phosphorylated based on several criteria including: (i) the pres-
ence of multiple keratin-charged isofoms upon analysis by two-dimensional gels, (ii) the presence of numerous serines/threonines (Ser/Thr), many of which are in proximity to arginine/lysine/proline residues that indicate potential kinase targets, and (iii) biochemical evidence for the phosphorylation of several of the epidermal keratins (21, 22). Here we identify Ser-35 as the major K19 phosphorylation site during interphase and show that mutation of this site interferes with K8/19 filament assembly in transfected NIH-3T3 cells. K19 phosphorylation is dynamic and is likely to involve multiple sites based on the increase in the number of K19 phosphopeptides and $^{32}$PO$_4$ incorporation upon treatment of cells with the Ser/Thr phosphatase inhibitor okadaic acid (OA). Although K19 and K18 type I keratins have some similarities in their phosphorylation properties, we show that they also have unique features that are likely to have functional correlates.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Reagents, and Metabolic Labeling—**HT29 (human), BHK (hamster), and NIH-3T3 (mouse) cells were obtained from the American Type Culture Collection (Manassas, VA). K19 cDNAs were kindly provided by Drs. Pierre Coulombe (The Johns Hopkins University) and Richard Eckert (Case Western Reserve University). Antibodies used in this work were monoclonal antibody (mAb) L2A1, which recognizes human K18 (23); rabbit antibody 8592, which recognizes human K8 and K18 (24); mAbs 4.62 and VM-13.2, which recognize human K19 and vimentin, respectively, from several species (Sigma). Other reagents used were OA (Alexis Corp., San Diego, CA), immobilized protein A-Sepharose and the BCA protein determination kit (Pierce), trypsin and chymotrypsin ( Worthington), enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech), $^{32}$PO$_4$-phosphoric acid (NEN Life Science Products).

HT29 cells were cultured using RPMI 1640 medium; BHK cells and NIH-3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C. BHK cell culture medium also included 100 μm methotrexate. For okadaic acid treatment, cells were cultured with 100 μM methotrexate. For manual Edman degradation, the major cellulose-containing $^{32}$PO$_4$-labeled spot was removed from cellulose plates followed by water extraction and lyophilization. The dried peptide was subjected to water extraction and lyophilization. The dried peptide was subjected to

**Immunoprecipitation—**Cells, with or without different treatments, were collected and washed with phosphate-buffered saline (PBS), pH 7.4. Cells were solubilized with 1% Nonidet P-40 in PBS (pH 7.4) containing 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 25 μg/ml aprotinin, and 0.5 μg/ml leupeptin (buffer A) (45 min, 4 °C), followed by pelleting (16,000 × g, 5 min, 4 °C). For some experiments, the residual pellet was subsequently solubilized with 1% Empigen BB (Emp) in buffer A (1 h, 4 °C) (25). The supernatants were used for immunoprecipitation with mAb L2A1 (covalently conjugated to protein A-Sepharose) or with mAb 4.62 (plus protein A G-Sepharose for 6 h at 4 °C) followed by washing five times with 0.5% Nonidet P-40 in PBS.

**Biochemical Analysis Methods—**Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels under nonreducing conditions (26). Proteins were stained with Coomassie Blue R-250. Two-dimensional gel electrophoresis was carried out as described (27) with isoelectric focusing in the first dimension (horizontal) and SDS-PAGE in the second dimension (vertical). The stoichiometry of K19 phosphorylation was estimated by densitometric scanning of Coomassie-stained isoelectric spots 1, 2, and 3 and then calculating the optical density ratio of phosphorylated spot 3 to the total phosphorylated and unphosphorylated K19 spots (1 + 2 + 3). For peptide mapping, K19 bands were isolated from Coomassie-stained gels followed by electroelution, concentration, and digestion with trypsin or chymotrypsin as described (28, 29). Peptide mapping was done using cellulose thin layer chromatography plates, with separation in the horizontal dimension by electrophoresis and in the vertical dimension by chromatography (28). For manual Edman degradation, the major cellulose-containing $^{32}$PO$_4$-labeled spot was removed from cellulose plates followed by water extraction and lyophilization. The dried peptide was subjected to manual Edman degradation for 5–15 cycles as described (29). The released materials after each cycle and the disks containing residual undegested peptide were counted.

**Construction of the K19 Serine Mutant and Cell Transfections—**K19 Ser-35 → Ala and Ser-10 → Ala mutants were cotransfected using a Transferrin™ kit (CLONTECH, Palo Alto, CA). The cDNA for human K19 was used as a template. Mutation was confirmed by sequencing followed by subcloning into the pMRRB101 mammalian expression vector with an hCMV promoter-directed expression. BHK or NIH-3T3 cells were cotransfected with wild type human K8 and one of three K19 constructs (wild type, Ser-35 → Ala, or Ser-10 → Ala). Given the phenotype that we observed with the Ser-35 K19 mutant, we also sequenced the entire K19 Ser-35 → Ala construct to confirm the absence of any other inadvertent mutations.

**Immunofluorescence Staining—**Transfected NIH-3T3 cells were cultured on coverslips for 3 days and then fixed in methanol (3 min, −20 °C). Fixed cells were blocked with PBS that contained 2% bovine serum albumin and a 1% cytoplasmic extract of nontransfected NIH-3T3 cells (15 min). Blocked cells were coincubated with mAb 4.62 (1:50 dilution) and rabbit antibody 8592 (1:1000 dilution) in PBS containing 2% bovine serum albumin for 30 min. After washing, cells were blocked again with PBS containing 2% bovine serum albumin and 2% goat serum for 15 min followed by incubating with Texas Red and fluorescein isothiocyanate-conjugated goat anti-mouse and goat anti-rabbit antibodies, respectively (30 min). Cells were visualized using a Nikon TE300 microscope coupled to a Bio-Rad MRC1024 confocal microscope. Quantitation of the keratin filament assembly phenotype was done by assigning the visualized cells (done by an independent investigator without prior knowledge of the transfection construct type to prevent bias) as having normal filaments, collapsed filaments (perinuclear), or short filaments. All fields on the slide were counted, and cells that did not double stain with the anti-K19 (mAb 4.62) and antibody 8592 were excluded.

**RESULTS**

**Characterization of K19 Phosphorylation—**We studied and characterized K19 phosphorylation to begin understanding its regulation and function. Metabolic labeling of colonic HT29 cells with $^{32}$PO$_4$ followed by immunoprecipitation of K8/19 showed incorporation of phosphate into both K8 and K19 (Fig. 1A). Phosphoamino acid analysis of $^{32}$PO$_4$-labeled K19 indicated that all of the incorporated radioactivity resided on serine (Fig. 1C), which is similar to what has been noted for the epidermal keratins (20, 21, 30) and for K8 and K18 (23, 31).

Previous phosphorylation studies of K8 and K18 showed that K8/18 phosphorylation increases during mitosis and that there is a phosphorylation gradient in that the membrane/cytosolic keratin fraction has a higher specific activity of phosphorylation as compared with the remaining cytoskeletal compartment (15, 19). The membrane/cytosolic-enriched fraction is efficiently extracted, although maintaining antrigency for immunoprecipitation, using the zwitte-rionic detergent Empigen (25). We tested if K19 has similar phosphorylation properties to K8 and K18. As shown in Fig. 1A, K19 manifests a phosphorylation gradient similar to K8 and K18 in that its phosphorylation increases in mitotically enriched cells (Fig. 1A, compare lane 2' with 1' and lane 3' with 4'). In addition, K19 (and K8) phosphorylation is increased in the Nonidet P-40 fraction as compared with the Emp fraction (Fig. 1A, compare lane 1' with 3' and lanes 2' with 4'). Furthermore, two-dimensional gel analysis (Fig. 1B) shows an acidic shift of K8 (isofrom 2) and K19 (isofrom 3) in K8/19 that is from the Nonidet P-40 fraction as compared with the Emp fraction. Similar acidic shifts of K19 and K8 were also noted in mitotically enriched cells after two-dimensional gel analysis (not shown). The stoichiometry of K19 phosphorylation in the Nonidet P-40 and Emp fractions under basal conditions is ~24 and 18%, respectively (see “Experimental Procedures”). Of note, K19 isofrom 2 does not appear to be phosphorylated as determined by the lack of incorporation of $^{32}$PO$_4$ upon metabolic labeling, which is incorporated into iso-
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Identification of Serine 35 as the Major K19 Phosphorylation Site—We used biochemical and molecular means to identify the K19 phosphorylation site(s). Metabolic labeling of K19 with $^{32}$PO$_4$ followed by protease digestion with trypsin or chymotrypsin and peptide mapping generated one major radiolabeled phosphopeptide (Fig. 2A). Manual Edman degradation of the purified phosphopeptide showed a single dominant radioactive serine that corresponds to the third amino acid position of the tryptic phosphopeptide (Fig. 2B) and the fourth amino acid position of the chymotryptic phosphopeptide (Fig. 2C). Inspection of the known K19 amino acid sequence (10, 11) and incorporation of the Edman degradation results and the predicted digestion sites of trypsin and chymotrypsin indicated that the major K19 phosphopeptide can only represent serine 10 ($^{32}$YRQSS) and/or serine 35 ($^{32}$FRAPS) phosphorylation. Mutation of K19 at Ser-10 or Ser-35 followed by transfection into BHK cells, radiolabeling with $^{32}$PO$_4$, and tryptic peptide mapping showed that Ser-35 is the major phosphorylation site of K19 (Fig. 3D, note absence of the Ser-35-containing spot).

Role of K19 Serine 35 in Keratin Filament Organization—Given that keratin phosphorylation can play a role in keratin filament organization, we asked if K19 Ser-35 has an effect on keratin filament assembly. As shown in Fig. 4, transfection of Ser-35 → Ala K19 into NIH-3T3 cells had a significant effect on filament assembly as compared with WT or with Ser-10 → Ala K19. As such, the Ser-35 K19 mutant resulted in a preponderance of cells that manifested perinuclear collapse (e.g., Fig. 4C) or short cytoplasmic filaments (e.g., Fig. 4D). For example, WT and Ser-10 → Ala K19 transfectants had 70 and 69%, respectively, of their cells with a normal-appearing filament organization, whereas the Ser-35 → Ala K19 transfectants had only 26% of their cells with normal-appearing filaments (Table I). Similar results were also obtained in transfected BHK cells (determined qualitatively, not shown). We chose NIH-3T3 cells for detailed quantitation of the immunofluorescence experiments instead of BHK cells (which were used primarily for protein isolation and peptide mapping experiments) because the latter have a significantly higher transfection efficiency (at the protein level) but far fewer cells that have normal-appearing filaments for all three constructs (particularly Ser-35 → Ala K19), thereby making assessment of an assembly phenotype in BHK cells inadequate. The keratin filament disruption that is noted in transfected NIH-3T3 cells does not affect the endogenous IF network (vimentin), as determined by double immunofluorescence staining using anti-vimentin and anti-keratin antibodies (not shown).

Relative Dynamic Nature of K8, K18, and K19 Phosphorylation—Our earlier observations of K8/18 phosphorylation showed that K8 phosphorylation is ~3–4-fold higher than K18 (19). The relatively similar basal phosphorylation of K8 and K19 (Fig. 1A, lanes 1’ and 3’) prompted us to formally examine the relative stoichiometry and dynamic nature of K8, K18, and K19 phosphorylation. We also compared the effect of the phosphatase I and IIA inhibitor OA (32) on K8, K18, and K19 phosphorylation. As shown in Fig. 5A (with quantitation in Table II), basal K19 and K8 phosphorylation are nearly equal, although basal K18 phosphorylation is significantly less than that of K8. Protein phosphatase inhibition by OA increases the phosphorylation of all keratins, but the increase in K19 phosphorylation is significantly less than that of K8 or K18 (Table II and Fig. 5A). The “relatively low” K19 hyperphosphorylation in the presence of OA is also evident by the small change in migration in SDS-PAGE gels as compared with K8 and K18 (Fig. 5A, hyperphosphorylated K8, K18, and K19 bands are highlighted by an asterisk). The results of these comparisons between K19 and K18 phosphorylation suggest that K19 in vivo phosphorylation is regulated very differently than K18 and that K19 in vivo dephosphorylation involves different phosphatase(s) than those involved in K8 or K18 dephosphorylation.

Although K19 has one major phosphorylation site basally

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**FIG. 1. Characterization of human K19 phosphorylation.** A, HT29 cells that are near confluent (enriched in the G$_0$/G$_1$ stage of the cell cycle) or growing in log phase (enriched in the S/G$_2$/M phases of the cell cycle) were labeled with $^{32}$PO$_4$ followed by sequential solubilization with Nonidet P-40 (NP40) and Emp as described under “Experimental Procedures.” The Nonidet P-40 and Emp cell lysates were then used to immunoprecipitate K19 with mAb 4.62 followed by separation by SDS-PAGE, Coomassie Blue staining, and autoradiography. Lanes 1–4 show the Coomassie stain of the corresponding lanes 1’–4’ of the radiograph. Note the increase in K8/19 phosphorylation in log phase versus confluent cells and the relatively higher specific activity of K19 phosphorylation in the Nonidet P-40 versus the Emp fraction. Cell cycle analysis of duplicate dishes of the near-confluent and log phase cells showed 37 and 47% of S/G$_2$/M cells, respectively (not shown). B, precipitates that are similar to those shown in A were obtained from non-$^{32}$PO$_4$-labeled HT29 cells followed by isoelectric focusing (IEF) in the horizontal dimension, SDS-PAGE in the vertical dimension, and then Coomassie staining of the gel. Note that the relative Coomassie intensity ratio of K19 isoform 3 (the only phosphorylated isoform under basal conditions) to the sum of K19 isoforms is higher in K19 that is isolated from the Nonidet P-40 fraction as compared with K19 that is isolated from the Emp fraction. C, HT29 cells were labeled with $^{32}$PO$_4$ (250 μCi/ml, 5 h) followed by solubilization with Emp, precipitation of K8/19, separation of K19 by SDS-PAGE, and phosphoamino acid analysis as described under “Experimental Procedures.” $^{32}$P, phosphothreonine; $^{32}$S, phosphoserine; $^{32}$T, phosphotyrosine; $^{32}$Y, phosphotyrosine. Dotted circles highlight the absence of detectable phosphothreonine and phosphotyrosine.
(i.e. Ser-35), it is likely that several other phosphorylation sites occur in vivo under different conditions. For example, human K19 has 38 serines (9–11), 20 of which are located in the head and tail domains, which are the most likely domains to undergo post-translational modifications based on what is known regarding IF protein phosphorylation (15, 16, 33, 34). Support for the presence of other K19 phosphorylation sites is evident after tryptic peptide mapping of K19 that is isolated from OA-treated 32PO4-labeled HT29 cells (Fig. 5B, compare panels a and b). Under these conditions, four other phosphopeptides become major radiolabeled peptides in addition to the Ser-35-containing phosphopeptide.

DISCUSSION

General Properties and Significance of K19 Phosphorylation—
Our results showed that K19 phosphorylation, under basal interphase conditions, can be accounted for by the predominant phosphorylation at a single, serine 35. This was confirmed by phosphoamino acid analysis (Fig. 1C), tryptic and chymotryptic peptide mapping (Fig. 2A), and manual Edman degradation (Fig. 2, B and C), which narrowed down the number of potential phosphorylation sites from the total of 38 K19 serines to 2 serines (Ser-10 or Ser-35). Confirmation of Ser-35 as the major K19 phosphorylation site was carried out by mutational analysis coupled with tryptic peptide mapping (Fig. 3). More importantly, mutation of K19 Ser-35 resulted in a filament assembly defect that consisted of perinuclear filament collapse and a propensity to form short filaments in transfected cells (Fig. 4 and Table I). The assembly defect that we observed differs from other K8 (Ser-73, Ser-431, and Ser-23) and K18 Ser-52 (35) phosphorylation sites that appear to have normal filament assembly upon cell transfection of their corresponding phosphorylation mutants (18, 19, 35).2 Notably, this assembly defect was unique to Ser-35 of K19 because mutation of Ser-10 in K19 did not have any noticeable impact on filament assembly (Fig. 4 and Table I). Defects in filament organization may be envisioned to occur at the level of assembly as noted for K19 Ser-35 or at the level of stimulus-induced filament reorganization as noted for K18 Ser-52 (35) and for nuclear lamin A Ser-22 (36).

Several potential explanations may be envisioned that could account for the observed assembly defect. The most attractive potential is the higher stoichiometry of K19 Ser-35 phosphorylation, in terms of single site phosphorylation, as compared with the only three other keratins whose phosphorylation has been studied in some detail. These keratins are: K8, which has similar overall phosphorylation stoichiometry to K19 but is

2 N-O. Ku and M. B. Omary, unpublished observations.
demonstrate normal-appearing filamentous arrays, whereas fested complete overlap with antibody 8592 staining (not shown). The figure shows only the anti-K19 antibody staining, which mani-
gognizes K8 and K18 as described under “Experimental Procedures.” stained with mAb 4.62 (anti-K19) and with antibody 8592, which rec-
morphology in the transfected cells is shown in Table I. show examples of perinuclear collapse (C) and short cytoplasmic fila-
lents (D) (highlighted by arrows). Quantitation of the keratin network morphology in the transfected cells is shown in Table I.

**Table I**

| Filament type | WT K19 | S35A K19 | S10A K19 |
|---------------|--------|----------|----------|
| Normal        | 70 ± 1 | 26 ± 2   | 69 ± 5   |
| Collapsed     | 19 ± 2 | 54 ± 7   | 17 ± 1   |
| Short         | 11 ± 1 | 20 ± 7   | 14 ± 4   |

 NIH-3T3 cells were transfected with WT K8 and one of the three K19 constructs: WT, Ser-35 → Ala, and Ser-10 → Ala. After 3 days, cells were double stained as described in Fig. 4 and then scored as having normal, collapsed, or short filaments. The percent ± S.E. is shown for each filament type and construct and represents an average of three experiments. The transfection efficiency was similar for each experiment and ranged from ~5 to 10%. The number of cells counted per experiment (and per construct) was 60–120.

divided nearly equally among 3–4 phosphorylation sites (29); K1, which also has multiple phosphorylation sites (8 serines and 1 threonine, Ref. 20); and K18, which has one major phosphorylation site but 3–4-fold lower phosphorylation stoichiometry. In this context, K19 Ser-35 phosphorylation could play a significant role in the in vivo assembly of K19-containing filaments possibly by impacting on the structure and/or the protein-protein interaction of K19. In addition, K19 appears to be basally significantly less stable than other type I keratins in terms of its ability to form stable complexes with type II keratins including K8 (37). This raises the possibility that K19 may be more susceptible to modulation by modifications such as phosphorylation changes. Other less likely possibilities that could account for the observed phenotype include an effect of the Ser-35 → Ala mutation, independent of phosphorylation, or differences in the expression level of the various transfected K19 constructs. To that end, K19 Ser-10 → Ala did not have any significant effect on keratin assembly (Fig. 4 and Table I), and the transfection efficiency of the different keratin constructs was similar as determined by immunofluorescence staining and immunoblotting of transfected cells (not shown). A role for phosphorylation in intermediate filament assembly has been demonstrated in vitro using purified keratins and a number of other IF proteins (reviewed in Refs. 15 and 16). This regulation can be modulated by affecting the solubility of keratins or by regulating keratin-keratin interaction or keratin interaction with other associated proteins. For example, in vitro phosphorylation of rat K8/18 by several kinases results in keratin filament disruption and increased solubility (38), and microinjection of cAMP into BHK cells results in IF reorganization (39). Furthermore, there are many examples of increased K8/18 solubility in association with increased keratin phosphorylation (reviewed in Ref. 16).
Aside from solubility, keratin phosphorylation can play an essential role in modulating the interaction with an associated protein. For example, K18 Ser-33 phosphorylation during mitosis regulates the binding of 14-3-3 proteins to K8/18 with consequent modulation of keratin filament arrangement (18, 19). At this stage, the mechanism of filament assembly modulation by K19 Ser-35 phosphorylation is not known, and we have not observed any easily detectable proteins that associate with K19 in a Ser-35 phosphorylation-dependent fashion. Notably, there is a solubility gradient of K8/19 that correlates with increased K8/19 phosphorylation. For example, the relative K19 phosphorylation in the more soluble Nonidet P-40 fraction is higher than that in the less soluble Emp (“cytoskeletal”) fraction (Fig. 1, A and B).

**Comparison of the Known Phosphorylation Properties among Type I Keratins**—The sequence context of K19 Ser-35 ( Thr/PAP ) is conserved in multiple species including human (10, 40, 41), bovine (9), mouse (42), and potoroo (43) but is unique when compared with other keratins. Based on the sequence context and known kinase consensus sequences (44), several kinases may be potential in vivo phosphorylating enzymes including cAMP-dependent protein kinase, protein kinase C, and calmodulin kinase II.

Although K19 phosphorylation may have features that are reminiscent of K18 phosphorylation, there are several distinguishing features that are highlighted by this study. The similarities among K18 and K19 phosphorylation are: (i) a single major interphase serine phosphorylation site (Ser-52 for K18, Ref. 35; and Ser-35 for K19, Fig. 3), (ii) a phosphorylation gradient with a relatively higher K18 and K19 phosphorylation in the cytosolic/membrane compartments (i.e. Nonidet P-40 solubilized) versus the remaining cytoskeletal compartment (i.e. Emp solubilized) (see Ref. 19 for K18 and Fig. 1 for K19), and (iii) the emergence of multiple phosphorylation sites upon phosphatase inhibition (see Ref. 45 for K18, and Fig. 5 for K19). The unique features of K19 phosphorylation, as compared with K18, are its higher basal phosphorylation state and the limited response to protein phosphatase types I and IIA inhibition. The relatively limited response of K19 phosphorylation to OA suggests that at least a significant portion of K19 phosphorylation is likely to be regulated by a unique set of kinases and phosphatases as compared with K5 and K18. This distinct regulation supports the hypothesis that K19 serves specific function(s) in epithelial cells, which is also supported by the unusual K19 structural feature of having a very short 13-amino acid tail domain as compared with other keratins (e.g. the next smallest keratin, K18, has a 39-amino acid tail, Refs. 46 and 47).

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