Identification of the Major Physiologic Phosphorylation Site of Human Keratin 18: Potential Kinases and a Role in Filament Reorganization

Nam-On Ku and M. Bishr Omary
Palo Alto Veterans Administration Medical Center, Palo Alto, CA 94304; and the Digestive Diseases Center, Stanford University School of Medicine, Stanford, California 94305-5487

Abstract. There is ample in vitro evidence that phosphorylation of intermediate filaments, including keratins, plays an important role in filament reorganization. In order to gain a better understanding of the function of intermediate filament phosphorylation, we sought to identify the major phosphorylation site of human keratin polypeptide 18 (K18) and study its role in filament assembly or reorganization. We generated a series of K18 ser→ala mutations at potential phosphorylation sites, followed by expression in insect cells and comparison of the tryptic 32P04-1abeled patterns of the generated constructs. Using this approach, coupled with Edman degradation of the 32P04-1abeled tryptic peptides, and comparison with tryptic peptides analyzed after labeling normal human colonic tissues, we identified ser-52 as the major K18 physiologic phosphorylation site. Ser-52 in K18 is not glycosylated and matches consensus sequences for phosphorylation by CAM kinase, S6 kinase and protein kinase C, and all these kinases can phosphorylate K18 in vitro predominantly at that site. Expression of K18 ser-52→ala mutant in mammalian cells showed minimal phosphorylation but no distinguishable difference in filament assembly when compared with wild-type K18. In contrast, the ser-52 mutation played a clear but nonexclusive role in filament reorganization, based on analysis of filament alterations in cells treated with okadaic acid or arrested at the G2/M stage of the cell cycle. Our results show that ser-52 is the major physiologic phosphorylation site of human K18 in interphase cells, and that its phosphorylation may play an in vivo role in filament reorganization.

Intermediate filaments (IF) consist of a broad family of nuclear (i.e., lamins) and cytoplasmic (e.g., keratins, neurofilaments [NF], vimentin, desmin) cytoskeletal proteins (reviewed in Steinert and Roop, 1988; Klymkowsky et al., 1989; Skalli and Goldman, 1991). The function(s) of the cytoplasmic IF remain largely unknown. However, recent identification of point mutations in a number of epidermal keratins as a likely cause of three skin diseases (reviewed in Fuchs and Coulombe, 1992; Compton, 1994) strongly support a structural/mechanical integrity role at least for the epidermal keratins (Coulombe et al., 1991). For the glandular epithelial keratins, a role in the integrity of fetal liver was demonstrated by a targeted null mutation in mouse keratin polypeptide 8 gene, with fetal lethality and extensive hepatic hemorrhage in most mice (Baribault et al., 1993). There is also evidence for keratins playing an important role in Xenopus gastrulation, based on injection of anti-keratin antibodies (Klymkowsky et al., 1992) or injection of antisense oligodeoxynucleotides into oocytes (Torpey et al., 1992). In the case of neurofilaments, overexpression of NF-L (Xu et al., 1993) and NF-H (Cote et al., 1993) in transgenic mice resulted in a disease resembling amyotrophic lateral sclerosis. In addition, NF-deficient axons have a small caliber as noted in the NF-deficient Japanese quail (Ohara et al., 1993) and in transgenic mice expressing an NF-β-galactosidase fusion protein (Eyer and Peterson, 1994). These findings suggest that NF may play a role in axonal caliber and neuronal transmission.

Although the function(s) of cytoplasmic IF is not fully understood, it is likely that IF are similar to other proteins in that at least some aspects of their function are regulated by posttranslational modifications they undergo. In the case of keratin polypeptide 8 and 18 (K8/18), which are the major keratin pair that is expressed in glandular epithelia, two potential regulatory posttranslational modifications are phosphorylation (Celis et al., 1983; Baribault et al., 1989; Klymkowsky et al., 1991; Yano et al., 1991; Chou and Omary, 1994).
1993) and glycosylation (Chou et al., 1992). In vitro phosphorylation of K8/18 using a number of kinases (Yano et al., 1991), or enhanced phosphorylation of a number of keratins in cells arrested in mitosis (Chou and Omary, 1993) or treated with the phosphatase inhibitor okadaic acid (Yat-sunami et al., 1993) are associated with disassembly and re-arrangement of the keratin filaments.

Phosphorylation of human K8 and 18 occurs on serine residues and increases dramatically after arresting cells at the G2/M stage of the cell cycle using anti-microtubule agents such as colcemid or nocodazole (Chou and Omary, 1993). In the case of K18, the phosphorylation occurs within the NH2-terminal 125 amino acids which include the head and proximal rod domains (Chou and Omary, 1993). Recently, we coexpressed K8 and K18 in Spodoptera frugiperda (Sf9) insect cells using recombinant baculovirus constructs and showed that the expressed keratins can assemble into disorganized filaments (Ku and Omary, 1994). We also showed that phosphorylation and glycosylation of K8/18 is highly conserved among insect and human tissue cultured cells, particularly for K18 (Ku and Omary, 1994). For example, K18 expressed in insect Sf9 cells was phosphorylated on serine within the first 125 amino acid residues and showed a tryptic phosphopeptide pattern that was nearly identical to that seen in human colonic HT29 cells. The tryptic phosphopeptide pattern of K18 in 32PO4-labeled HT29 and K18-recombinant baculovirus infected Sf9 cells showed several spots consistent with phosphorylation at multiple sites. However, one spot predominated as a major phosphopeptide in both cell types (Ku and Omary, 1994). One noted difference between insect and human tissue culture cells was in the K8:K18 phosphorylation ratio, such that phosphorylation was K8>18 in HT29 cells and K18>K8 in Sf9 cells (Ku and Omary, 1994).

In this report, we first show that phosphorylation of K18 and K8 in HT29 tissue culture cells is very similar to what is seen in ex vivo isolated normal colonic tissues. We then use a mutational approach of K18 coupled with expression in insect cells to identify ser-52 as the major phosphorylation site of K18 in interphase cells. A ser-52→alanine (ala) mutation did not alter filament assembly in transfected mammalian cells, but did interfere with filament reorganization in cells arrested at the G2/M stage of the cell cycle or cells treated with okadaic acid.

**Materials and Methods**

**Cell Culture, Tissues, and Reagents**

HT29 (human colon), NIH-3T3 (mouse fibroblast), NMuLi (mouse liver) (American Type Culture Collection, Rockville, MD), and SV13 cells (Clone L1, vimentin positive human adrenal, kindly provided by Dr. R. Evans, University of Colorado, Denver, CO) were grown in media recommended by the providers. Rectal biopsies were obtained from patients undergoing routine colonoscopy for polyp screening under a protocol approved by the Medical Committee for the Protection of Human Subjects on Research at Stanford University. The radiochemicals uridine diphosphate (UDP) -[3-3H]glucosamine, orthophosphate (32PO4), and F6-F1ATP (3,000 Ci/mmol) were purchased from Du Pont-New England Nuclear. Other reagents used were: bovine milk galactosyltransferase, myelin basic protein (MBP), and colcemid (Sigma Chem. Co., St. Louis, MO); BaculoGold transfection kit and Sf9 insect cells (PharMingen, San Diego, CA); the CDNA for wild-type (WT) human K8 and K18 were kindly provided by J. F. Strauss (Yamamoto et al., 1990) and R. Oshima (Oshima et al., 1986), respectively. The S6 and calcium/calmodulin-dependent (CAM) kinases were gifts from James Mailer (University of Colorado, Denver, CO) and Howard Schulman (Stanford University, Palo Alto, CA), respectively, and protein kinase C (PKC) α and ε were obtained from Berta Strulovic (Syntex Research, Palo Alto, CA).

**Construction of Mutants and Baculovirus Recombinants**

The cDNA for K8 and K18 were subcloned into the pBluescript SK+ plasmid. Site-directed mutagenesis to generate serine (ser)→ala mutants were carried out using a Transformer® kit (Clontech, Palo Alto, CA). This method is based on annealing two oligonucleotide primers, one which introduces the desired mutation and the second which mutates a restriction site unique to the plasmid for selection (Deng and Nickoloff, 1992). All primers contained 1-3 mutations with 8-10 nucleotides flanking the first mutation on either end. Mutations of nonconsecutive serines were generated using multiple primers, and all mutations were confirmed by sequencing the mutagenized regions of both strands. Mutants were subcloned into the pVL392 vector for expression in Sf9 cells, or downstream of the hCMV promoter in pMRB101 mammalian expression vector (Stephens and Cockett, 1989). Generation of the baculovirus recombinants was done exactly as described (Ku and Omary, 1994).

**Transient and Stable Transfection**

Transient transfection was done using LipofectAMINE™ liposomes (GIBCO BRL). Selection of stable transfectants was done by growing cells in the presence of 250 μg/ml xanthine, 15 μg/ml hypoxantheme, 10 μg/ml thymidine, 2 μg/mlaminopterin, 25 μg/ml mycophenolic acid, 150 μg/ml glutamine, and fetal calf serum dialyzed against 0.15 M NaCl. Transfectants were then sub-cloned by limiting dilution. Sodium butyrate was used to enhance the expression of K8/18 in stably transfected NIH-3T3 cells (Gorman et al., 1985), before labeling cells with 32PO4. For this, cells were incubated with 10 mM sodium butyrate (using 1 M stock solution) for 16-18 h before (and during) 32PO4 labeling. It resulted in a 3-5-fold increase in K8/18 expression without affecting cell viability, if phosphorylation or organization (not shown).

**Immunofluorescence Microscopy**

Transfected mammalian cells, or Sf9 cells infected with K8/18 recombinant baculovirus constructs were grown on coverslips. Alternatively, transfected cells were treated with okadaic acid (1 μg/ml) for 30-60 min, or with colcemid (0.5 μg/ml) for 3 h followed by fixation of floaters and easily released cells and transferring to slides using a Cytospin (700 rpm, 6 min). Cells were then fixed in methanol (3 min, -20°C), rinsed with PBS then PBS containing 2.5% BSA. After washing, cells were incubated with monoclonal anti-K8/18 antibody (termmed L2A1; Chou and Omary, 1991) in PBS containing 2.5% BSA and 10% (vol/vol) goat serum and 10% (vol/vol) nontransfected cell cytoplasmic extract. For quantitation of filament reorganization, cells from each treatment were counted (102-278 cells/treatment condition of a cell transfectant). Counted cells were categorized as having an exclusive punctate pattern or a mixed punctate/filamentous pattern (grouped as disrupted) or cells that lack any punctate pattern (grouped as normal filaments). The percent reorganization was quantitated as number of cells with disrupted filaments/number of cells with normal + disrupted filaments × 100. Photos were taken using black and white Kodak TMAX 400 film. The mitotic index (%G2/M cells) was determined using propidium iodide staining as described (Chou and Omary, 1993).

**Radiolabeling**

Human colon biopsies and tissue culture cells were labeled with 32PO4 for 3-6 h (250 μCi/ml) in phosphate-free RPMI 1640 medium containing 10% dialyzed fetal calf serum. K8/18 were then isolated by immunoprecipitation or high salt extraction as described below. Analysis of K8/18 glycosylation was done by labeling accessible K8/18 terminal GlcNAc's by galactosylating K8/18 immunoprecipitates using UDP-[3H]galactose and galactosyltransferase exactly as described (Ku and Omary, 1994).
Keratin Isolation

Immunoprecipitation was used to analyze K8/18 from cells labeled metabolically with $^{32}$P0$_4$, or to isolate keratins for further glycosylation analysis or kinase assays. For this, mammalian and insect cells were solubilized in 1% NP-40 in PBS containing 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (added fresh), 10 µM ioepeptin, 10 µM pepstatin, and 25 µg/ml aprotinin (buffer A) (45 min, 4°C). After removal of nuclei (10,000 g; 10 min), cell lysates were incubated with anti-K8/18 MAb conjugated to Sepharose beads (45 min, 4°C) followed by washing. Alternatively, keratins were isolated from $^{32}$P0$_4$-labeled cells using high salt extraction (Achstetter et al., 1986) exactly as described (Chou et al., 1993). K8/18 was also isolated from normal colonic biopsies by homogenization of the labeled tissue fragments in buffer A using a Dounce homogenizer, followed by centrifugation and immunoprecipitation.

Biochemical Studies

Tryptic glyco- and phosphopeptide mapping was carried out as described (Chou et al., 1993). In the case of $^{32}$P0$_4$-labeled K8/18, individual keratins were isolated after preparative SDS-PAGE by cutting K8 or K18 bands visualized by brief Coomassie blue staining followed by electroelution then acetone precipitation. Keratin precipitates were treated with trypsin (375 µg/ml in 80 µl of 50 mM NH$_4$HCO$_3$) for 16 h followed by a lyophilization then analysis in the horizontal dimension by electrophoresis (pH 1.9 buffer) (Boyle et al., 1991) and in the vertical dimension by chromatography.

Manual Edman degradation was carried out as described (Boyle et al., 1991) using trypptic digests of K18 that were purified from in vivo $^{32}$P0$_4$-labeled HT29 or Sf9 cells. Individual peptides were not separated before modification with phenyl isothiocyanate in the case of asynchronously growing HT29 cells, since >90% of the radioactivity contained in the $^{32}$P0$_4$-labeled trypptic digest was in the ser-52-containing peptide that was being studied. For analysis of the ser-52-containing phosphopeptide from G2/M arrested HT29 cells, the specific peptide was scraped from six cellulose chromatography plates. The $^{32}$P0$_4$-labeled peptide was then extracted using water (~15,000 cpm) followed by manual Edman degradation.

In Vitro Kinase Assays

Phosphorylation of K8/18 immunoprecipitates obtained from HT29 cells was carried out using rat brain PKC$_{a}$ and e (Omary et al., 1992), CAM kinase (Hanson and Schulman, 1992), and Xenopus ribosomal S6 kinase II (Erikson et al., 1991). Immunoprecipitates were first boiled for 2 min to remove endogenous kinase activity. S6 kinase reactions were done in: 20 mM Hepes (pH 7), 3.3 mM 2-mercaptoethanol, and 10 mM MgCl$_2$. Other kinase reactions were done in 50 mM Pipes (pH 7), 5 mM MgCl$_2$, 10 mM MnCl$_2$ which also contained: 5 mM CaCl$_2$, 5 µg/ml calmodulin (for CAM kinase); phorbol 12-myristate 13-acetate (1 µM), diacylglycerol (4 µg/ml), phosphatidylserine (40 µg/ml) (for PKC$_{a}$ and e). In addition, the PKC$_{e}$ reaction contained 1 mM EGTA. All reactions were done in a total volume of 25 µl that also contained 10 µM ATP and 5 µCi of [$^{32}$P]ATP. After addition of the respective kinases (10 min, 22°C), 25 µl of 10% glycerol, 4% SDS sample buffer was added (Laemmli, 1970). Samples were then analyzed by SDS-PAGE and autoradiography, or tryptic peptide mapping of purified K18.

Results

Phosphorylation of K8/18 in Sf9 and HT29 Cells Correlates with Phosphorylation in Normal Human Tissues

We recently showed that phosphorylation of K8 and K18 expressed in Sf9 insect cells was very similar to that noted in HT29 human colonic tissue cultured cells, particularly for K8 (Ku and Omary, 1994). Before embarking on the identification of K8 and K18 phosphorylation sites in Sf9 and HT29 model systems, we sought to confirm that the labeled phosphopeptides in HT29 cells reflect the physiologically labeled sites in normal tissues. As shown in Fig. 1 A, K8/18 can be phosphorylated after labeling biospies of normal human colon ex vivo with $^{32}$P0$_4$. Incorporation of $^{32}$PO$_4$ into the type II keratin (i.e., K8) was higher than the type I keratin (i.e., K18) as seen in HT29 cells (Chou and Omary, 1993), Xenopus oocytes (Klymkowsky et al., 1991) and rat hepatocytes.

Figure 1. Comparison of human K8 and K18 phosphorylation in HT29 cells and normal colonic biopsies. (A) Normal mucosal rectal biopsies were obtained from two patients undergoing routine colonoscopy for polyph screening. After rinsing in RPMI 1640 medium, biopsies were cut into small pieces then labeled with $^{32}$PO$_4$ for 3 h, followed by detergent solubilization and immunoprecipitation using anti-K8/18 MAb (lane b) or normal mouse ascites (lane a). The radiograph was obtained by exposing the Coomassie-stained gel shown. Arrowhead indicates migration of antibody used for immunoprecipitation. (B) K8 and K18 were individually purified (using SDS-PAGE followed by cutting out of individual bands and electrophoresis) from HT29 cells that were radiolabeled with phosphate, or from the labeled normal colonic biopsies used in A. Purified K8 and K18 were digested with trypsin for peptide mapping. Electrophoresis and chromatography were in the horizontal and vertical directions, respectively. Arrowheads correspond to origin of sample loading. Mixture maps (c and f) contained tryptic digest a + b and d + e, respectively (1,000 cpm from each tryptic digest). Numbered arrows correspond to prominent peptides.
cytes (Yano et al., 1991). Comparison of the tryptic phosphopeptides of K8 and K18 isolated from normal human colon versus tissue cultured cells showed significant identity (Fig. 1 B). For example, all the numbered peptides for K8 were shared and similar in labeling intensity (Fig. 1 B, c) except peptide 5 which was labeled primarily in normal colon. Similarly, the minor labeled peptides 2–5 of K18 were shared although more prominent in HT29 cells, with peptide 1 being the major labeled species in normal colon and HT29 cells (Fig. 1 B) and in Sf9 cells (Ku and Omary, 1994; and see Fig. 3 below).

Identification of ser-52 as the Major Phosphorylation Site of K18

In order to identify the major phosphorylation site of K18, we used a systematic approach of generating a series of K18 constructs that contain ser→ala mutations at potential phosphorylation sites. That approach was based on our biochemical data showing that phosphorylation of K18 in Sf9 cells (Ku and Omary, 1994) and HT29 cells (Chou and Omary, 1993) was localized to the NH2-terminal 125 amino acids after digestion of 32PO4-labeled K18 with HBr/HCl at tryptophan (trp) residues. As shown in Fig. 2, the NH2-terminal 125–amino acid domain of K18 contains 20 serine potential phosphorylation sites. The phosphorylation in Sf9 cells of K18 constructs encompassing mutations of the first 15 residues are shown in Fig. 3 A. Constructs b–g contained point mutations of the sequentially numbered serines shown in Fig. 2, and constructs h and i contained scattered point mutations. Immunoprecipitation of K8/18 from in vivo 32PO4-labeled Sf9 cells expressing the various constructs showed that constructs g and i had minimal phosphorylation of K18 as compared with the other constructs (Fig. 3, radiograph), despite having equal levels of expressed protein (Fig. 3 A, Coomassie stain).

The tryptic phosphopeptide map of K18 constructs (Fig. 3 B) showed that the major phosphopeptide indicated by an arrow in a is seen in all the constructs except g and i. By using the constructs that contain the major phosphopeptide to eliminate potential sites, it can be inferred that the 15th serine (i.e., ser-52 in Fig. 2) is the likely major site of K18 phosphorylation. This was confirmed by constructing individual ser-50 and ser-52 point mutations which are contained within the same tryptic peptide (Fig. 4). Of note, the ser-50 point mutation decreased the overall phosphorylation of K18 nearly fourfold whereas the ser-52 point mutation decreased...
Ser-52 is the major phosphorylation site of K18. Baculovirus-K18 constructs that contain wild-type K18, or individual ser-50→ala or ser-52→ala mutants (which correspond to the consecutively numbered serine 14 and 15 in Fig. 2) were generated and used with WT K8 to coinfect Sf9 cells for 4 d. Cells were then labeled with $^{32}$PO$_4$ followed by solubilization and immunoprecipitation of K8/18 (A) or analysis of K18 from each of the constructs by tryptic peptide mapping (B).

resulted in essentially total abolishment of K18 phosphorylation (Fig. 4). Abolishing most of the phosphorylation of K18 via the ser-52→ala mutation did not affect the phosphorylation level of K8 (Fig. 4 A) or the peptide map pattern of K8 (not shown).

In order to differentiate between partial phosphorylation at ser-50 versus a ser-50→ala induced conformational change that decreases the efficiency of phosphorylation at ser-52, we performed manual Edman degradation of K18 tryptic phosphopeptides. As shown in Fig. 5, Edman degradation of K18 tryptic phosphopeptides, which contained the peptide 5°STSFR as the primary phosphopeptide, resulted in release of phosphate after the third cycle. Therefore, ser-52, being the third residue of the 5°STSFR tryptic peptide, is the major phosphorylation site of K18. The presence of a single phosphorylation site in the peptide 5°STSFR was also supported by finding that WT K18 and the ser-50 mutants have identical pI and an identical migration of the $^{32}$PO$_4$-labeled isoelectric forms (not shown).

We also compared the glycosylation of wild type K18, ser-50→ala and ser-52→ala K18 mutants and found that all the constructs had similar glycosylation levels in K8 and K18 when expressed in Sf9 cells, as determined by in vitro galactosylation (not shown). In addition, the tryptic glycopeptide pattern of the three K18 constructs was also very similar and no effect of the mutations on the tryptic glycopeptide pattern of coexpressed wild-type K8 was noted (not shown).

**Potential Kinases Involved in K18 ser-52 Phosphorylation**

The sequence containing ser-52 is part of a consensus sequence RXXXSR (see Fig. 2) for PKC, and RXXS for S6 and CAM kinases (Pearson and Kemp, 1991). We tested the in vitro phosphorylation of K18 (immunoprecipitated from HT29 cells) using purified CAM and S6 kinases, PKCα and PKCe. As shown in Fig. 6 a, K18 was phosphorylated by all the tested kinases except PKCα. As a control, phosphorylation of MBP by the different kinases showed that while PKCα did not efficiently phosphorylate K18, it did phosphorylate MBP (Fig. 6 b). Analysis of the tryptic phosphopeptides of K18 labeled in vitro by the indicated kinases (Fig. 6, e–g) showed a very similar pattern to the in vivo phosphorylation shown in Fig. 1. Identity of peptides 1–3 shown in Fig. 6, e–g to peptides 1–3 shown in Fig. 1 was confirmed by analysis of peptide maps obtained after mixing K18 isolated from in vivo phosphate-labeled HT29 cells with K18 labeled in vitro by each of the kinases (not shown). This indicates that CAM and S6 kinases as well as PKCe are able to in vitro phosphorylate the ser-52 containing peptide of K18.

**Expression of Wild-type and ser-52→ala K18 in Mammalian and Insect Cells and Analysis of Keratin Filament Assembly and Reorganization**

We examined the effect of the ser-52→ala mutation of K18 on filament assembly in a number of expression cell systems using immunofluorescence. As shown in Fig. 7, mutant and wild-type K18 manifested similar assembly patterns in Sf9 cells, and in three mammalian cell lines containing endogenous vimentin (mouse NIH 3T3 and human SW13) or mouse K8/18 (NMuLi). Similar assembly patterns were also
Phosphorylation of K18 by CAM and S6 kinases and PKCe. (a–d) An in vitro kinase assay, with the indicated kinases, was carried out using as a substrate MBP or four equivalent immunoprecipitates of K8/18 isolated from HT29 cells as described in Materials and Methods. The radiograph was obtained by exposure of the Coomassie blue panel shown. (e–g) K18 was purified using preparative SDS-PAGE from K8/18 immunoprecipitates labeled in vitro with CAM or S6 kinases, or PKCe. After electrophoresis, the individual K18 bands, tryptic peptide mapping was carried out as described in Materials and Methods. Arrowheads indicate origin where samples were spotted. Unlabeled arrow in g shows a labeled peptide that is only weakly seen after in vivo labeling (Fig. 1).

Figure 6. Phosphorylation of K18 by CAM and S6 kinases and PKCe. (a–d) An in vitro kinase assay, with the indicated kinases, was carried out using as a substrate MBP or four equivalent immunoprecipitates of K8/18 isolated from HT29 cells as described in Materials and Methods. The radiograph was obtained by exposure of the Coomassie blue panel shown. (e–g) K18 was purified using preparative SDS-PAGE from K8/18 immunoprecipitates labeled in vitro with CAM or S6 kinases, or PKCe. After electrophoresis, the individual K18 bands, tryptic peptide mapping was carried out as described in Materials and Methods. Arrowheads indicate origin where samples were spotted. Unlabeled arrow in g shows a labeled peptide that is only weakly seen after in vivo labeling (Fig. 1).

Phosphorylation of K18 by CAM and S6 kinases and PKCe. (e–g) K18 was purified using preparative SDS-PAGE from K8/18 immunoprecipitates labeled in vitro with CAM or S6 kinases, or PKCe. After electrophoresis, the individual K18 bands, tryptic peptide mapping was carried out as described in Materials and Methods. Arrowheads indicate origin where samples were spotted. Unlabeled arrow in g shows a labeled peptide that is only weakly seen after in vivo labeling (Fig. 1).

We also studied the effect of the ser-52 K18 mutation on K8/18 filament reorganization in tissue culture cells. This is based on in vivo and in vitro evidence indicating that phosphorylation of IF is likely to play a role in filament reorganization (reviewed by Eriksson et al., 1992a). This was done in two ways. First we induced a hyperphosphorylated state in K8/18 transfected cells by short-term treatment with okadaic acid, a phosphatase type 1 and 2A inhibitor (Bialojan and Takai, 1988), which results in reorganization of IF (Eriksson et al., 1992b; Sacher et al., 1992; Lee et al., 1992) including keratins (Yatsunami et al., 1993) in a number of cell types examined. As shown in Fig. 8, okadaic acid treatment of wild-type K8/18 transfected into NIH-3T3 cells (and SW13 cells, not shown) resulted in rearrangement of the transfected filament network into small and large aggregate dots, or dots and short filaments (a and c). In contrast, treatment of ser-52→ala K18/WT K8→transfected cells with okadaic acid resulted in a less filament-destructive phenotype manifested by many cells showing normal appearing filaments and some cells showing fine dots (Fig. 8, e and g). Longer treatment with okadaic acid (>1 h) resulted in complete rearrangement of K8/18 filaments in WT and ser-52→ala transfectants (not shown). A second approach that we utilized was to arrest WT K18 and K18 ser-52→ala transfected SW13 cells at the G2/M stage of the cell cycle with colcemid, then examine the extent of filament rearrangement. As shown in Fig. 8 (b, d, f, and h), a similar pattern to that seen with okadaic acid was observed in that the ser-52 K18 mutant did not rearrange as well as the WT K18 transfectant. Quantitation of the cells containing disrupted filaments from a representative experiment showed that okadaic acid treated cells had 47 and 27% of the cells with disrupted filaments (for WT and ser-52 mutant, respectively) and colcemid G2/M arrested cells had 63 and 35% of the cells with disrupted filaments (for WT and ser-52 mutant, respectively). Although there was some variability in the percent of cells with rearranged filaments between experiments, the ratio of percent filament rearrangement in WT versus ser-52 mutant K18 transfectants was always reproducible (1.96 ± 0.14, [n = 4], and 2.15 ± 0.78 [n = 4] for colcemid and okadaic acid treated cells, respectively). This near twofold effect was also confirmed when WT and ser-52→ala K18 transfectants (okadaic acid or colcemid treated) were analyzed in a double-blinded manner (not shown). We did not analyze G2/M arrested NIH-3T3-transfected cells since their filaments were not disrupted after colcemid treatment (not shown).

Phosphorylation of Wild-type and ser-52→ala K18 Expressed in NIH-3T3 Cells

The ser-52→ala mutation of K18, expressed in S9 cells, resulted in near total abolishment of K18 phosphorylation (Fig. 4), which predicts that a similar pattern should occur if the K18 mutant is expressed in mammalian cells. This prediction was tested and as shown in Fig. 9, WT K8/18 were phosphorylated in the expected pattern (i.e., K8>K18 32P04-labeling intensity) after expression in NIH-3T3 cells (lane a), but the phosphorylation of the ser-52→ala K18 mutant was barely detectable (lane b). This indicates that mutation of the major phosphorylation site of K18 in mammalian cells abolishes most of K18 in vivo phosphorylation, and supports our biochemical and molecular characterization. Analysis of the phosphorylation of WT K8/18 in the same transfected cells treated with okadaic acid showed that K8 and K18 become hyperphosphorylated with K18 having nearly similar phosphorylation levels to K8 (lane c). A similar preferential hyperphosphorylation of K18 was also noted in HT29 cells treated with okadaic acid (not shown). In contrast, okadaic acid treated cells transfected with ser-52→ala K18 showed that K8 became relatively more phosphorylated than K18 (lane d), indicating that the ser-52 mutation prevented some phosphorylation of K18 but that other sites also become phosphorylated. Similar results were noted when wild-type K18 and the ser-52 mutant were expressed in SW13 cells that lack vimentin (not shown). We were unable to analyze in vitro assembly (using negative staining and electron microscopy) of K8/18 purified from the Sf9 cell expression system, due to lack of filament formation of the wild-type proteins despite normal filament formation of K8/18 isolated in a similar manner from HT29 cells (not shown). Lack of in vitro filament assembly of Sf9 produced K8/18 could be related to interfering species that may copurify with the individual keratins or to the altered phosphorylation ratio of K8/K18 in insect cells as compared with mammalian cells (Ku and Omary, 1994).

We also studied the effect of the ser-52 K18 mutation on K8/18 filament reorganization in tissue culture cells. This is based on in vivo and in vitro evidence indicating that phosphorylation of IF is likely to play a role in filament reorganization (reviewed by Eriksson et al., 1992a). This was done in two ways. First we induced a hyperphosphorylated state in K8/18 transfected cells by short-term treatment with okadaic acid, a phosphatase type 1 and 2A inhibitor (Bialojan and Takai, 1988), which results in reorganization of IF (Eriksson et al., 1992b; Sacher et al., 1992; Lee et al., 1992) including keratins (Yatsunami et al., 1993) in a number of cell types examined. As shown in Fig. 8, okadaic acid treatment of wild-type K8/18 transfected into NIH-3T3 cells (and SW13 cells, not shown) resulted in rearrangement of the transfected filament network into small and large aggregate dots, or dots and short filaments (a and c). In contrast, treatment of ser-52→ala K18/WT K8→transfected cells with okadaic acid resulted in a less filament-destructive phenotype manifested by many cells showing normal appearing filaments and some cells showing fine dots (Fig. 8, e and g). Longer treatment with okadaic acid (>1 h) resulted in complete rearrangement of K8/18 filaments in WT and ser-52→ala transfectants (not shown). A second approach that we utilized was to arrest WT K18 and K18 ser-52→ala transfected SW13 cells at the G2/M stage of the cell cycle with colcemid, then examine the extent of filament rearrangement. As shown in Fig. 8 (b, d, f, and h), a similar pattern to that seen with okadaic acid was observed in that the ser-52 K18 mutant did not rearrange as well as the WT K18 transfectant. Quantitation of the cells containing disrupted filaments from a representative experiment showed that okadaic acid treated cells had 47 and 27% of the cells with disrupted filaments (for WT and ser-52 mutant, respectively) and colcemid G2/M arrested cells had 63 and 35% of the cells with disrupted filaments (for WT and ser-52 mutant, respectively). Although there was some variability in the percent of cells with rearranged filaments between experiments, the ratio of percent filament rearrangement in WT versus ser-52 mutant K18 transfectants was always reproducible (1.96 ± 0.14, [n = 4], and 2.15 ± 0.78 [n = 4] for colcemid and okadaic acid treated cells, respectively). This near twofold effect was also confirmed when WT and ser-52→ala K18 transfectants (okadaic acid or colcemid treated) were analyzed in a double-blinded manner (not shown). We did not analyze G2/M arrested NIH-3T3-transfected cells since their filaments were not disrupted after colcemid treatment (not shown).

Phosphorylation of Wild-type and ser-52→ala K18 Expressed in NIH-3T3 Cells

The ser-52→ala mutation of K18, expressed in S9 cells, resulted in near total abolishment of K18 phosphorylation (Fig. 4), which predicts that a similar pattern should occur if the K18 mutant is expressed in mammalian cells. This prediction was tested and as shown in Fig. 9, WT K8/18 were phosphorylated in the expected pattern (i.e., K8>K18 32P04-labeling intensity) after expression in NIH-3T3 cells (lane a), but the phosphorylation of the ser-52→ala K18 mutant was barely detectable (lane b). This indicates that mutation of the major phosphorylation site of K18 in mammalian cells abolishes most of K18 in vivo phosphorylation, and supports our biochemical and molecular characterization. Analysis of the phosphorylation of WT K8/18 in the same transfected cells treated with okadaic acid showed that K8 and K18 become hyperphosphorylated with K18 having nearly similar phosphorylation levels to K8 (lane c). A similar preferential hyperphosphorylation of K18 was also noted in HT29 cells treated with okadaic acid (not shown). In contrast, okadaic acid treated cells transfected with ser-52→ala K18 showed that K8 became relatively more phosphorylated than K18 (lane d), indicating that the ser-52 mutation prevented some phosphorylation of K18 but that other sites also become phosphorylated. Similar results were
Discussion

Ser-52 Is the Major Physiologic Phosphorylation Site of K18 in Interphase Cells

The phosphorylation of human K18 appears to be highly conserved in that similar peptides are phosphorylated when analyzed in an insect cell expression system, human colonic tissue cultured or normal cells. For example, the phosphorylated peptides of K18 in normal intestine are nearly identical to those in human colonic tissue culture cells, with one predominant radiolabeled peptide (Fig. 1). This extends our recent findings showing that phosphorylation of K8 and K18 in HT29 cells and SF9 insect cells is also similar (Ku and Omary, 1994), and indicates that the phosphorylation of K8/18 expressed in SF9 cells is physiologically relevant in terms of the modified peptides. The baculovirus-SF9 K8/18 expression system allowed us to generate a panel of K18 constructs in large quantities that permitted biochemical studies. The generated K18 constructs contained multiple serial mutations at potential phosphorylation sites that allowed identification of the ^35S tryptic peptide as the major phosphopeptide of K18 (Fig. 3). The precise location of the phosphorylation site was verified using Edman degradation of tryptic peptides of ^32P-labeled K18 (Fig. 5) and by generating specific point mutations (Fig. 4). In addition, the ser-52 K18 mutant expressed in mammalian cells was essentially not phosphorylated when compared to the wild-type expressed protein (Fig. 9). These data support the conclusion that ser-52 is the major physiologic phosphorylation site of K18 in interphase cells.

It is possible that the ser-52 mutation, which abolished
most of K18 phosphorylation, could have caused a conformational change that resulted in masking another presumed major phosphorylation site. Such a scenario is unlikely for several reasons. First, biochemical data based on cleavage at trp-125 (Fig. 2) showed that most of K18 phosphorylation is within the NH2-terminal 125-amino acid domain which contains 20 serines (Chou and Omary, 1993). As a complement to the analysis of the first 15 serines shown in Fig. 3, analysis of ser→ala mutations of the five remaining serines showed a similar phosphorylation level to wild-type K18 (not shown). Second, preliminary mass spectrometry data of a tryptic digest of purified K18 yielded a prominent mass spectral peak that corresponded to the mass of a single phosphate plus the 49RSTSFR peptide (not shown). Third, a significant conformational change by the ser-52→ala mutation is unlikely, at least to the extent that it did not appreciably alter filament assembly (Fig. 7). Along the same line, the 75% reduction in K18 phosphorylation in the ser-50→ala mutant is likely related to a decrease in the overall phosphorylation of K18 because of the nearby location of ser-50 and ser-52. Of note, the ser-50→ala K18 mutant showed normal assembly, as determined by immunofluorescence staining, after transfection into NIH-3T3 cells (not shown).

The 49RSTSFR sequence of human K18 appears to be unique in terms of being absent from other keratin head-domain sequences that we have examined from sequence data banks. It is also not present in Endo B, the mouse kera-
Relationship between Glycosylation and Exclusiveness of each Modification

Glycosylation and phosphorylation is that the K8 and K18 molecules that are phosphorylated are not glycosylated and vice versa. This is based on metabolic labeling of HT29 cells in which has 89.7% amino acid sequence identity with human K18 (Oshima et al., 1986). It remains to be determined if ser-51 in the sequence *RSVWGG of mouse Endo B is functionally equivalent to ser-52 in human K18.

Functions to Consider for ser-52 Phosphorylation

Several potential functions for IF phosphorylation can be considered, including the regulation of IF assembly, disassembly, and dynamic rearrangements, interaction with other IF-associated proteins, glycosylation, and signal transduction. Of these potential functions, the role of phosphorylation in IF disassembly and reorganization is the best studied. For example, in vitro phosphorylation of vimentin (Inagaki et al., 1987), desmin (Inagaki et al., 1988), K8/18 (Yano et al., 1991), NF (Gonda et al., 1990), α-internexin (Tanaka et al., 1993), and nuclear lamins (Peter et al., 1991) resulted in IF disassembly, and microinjection of cAMP-dependent protein kinase into BHK cells (Lamb et al., 1989) caused rearrangement of the studied IF. Also, mitotic arrest of BHK cells caused hyperphosphorylation of vimentin and desmin in association with IF disassembly (Chou et al., 1989), and mitotic arrest of HT29 cells resulted in a similar change in K8/18 (Chou and Omary, 1993). Interestingly, phosphorylation of the studied IF proteins occurs in the head, tail, or head/tail domains as shown for vimentin and desmin (Evans, 1988), K1 (Steinert, 1988), NF (Julien and Mushynski, 1983; Nixon and Sihag, 1991), and K18 (Chou and Omary, 1993). These are the domains that appear to be important in IF assembly and dynamics (reviewed by Coulombe, 1993; Stewart, 1993; Heins and Aebi, 1994).

Potential Kinases Involved in K18 Phosphorylation

The consensus sequence RXXXSXR for PKC, S6, and CAM kinases corresponds to the sequence *RSTSRFR containing ser-52 of K18. Our in vitro phosphorylation data showed that the ser-52 containing peptide was a major phosphopeptide that was phosphorylated by all three kinases (Fig. 6). In vivo phosphorylation of K8/18 in HT29 cells was previously shown to be calcium independent, based on unaltered K8/18 phosphorylation in the presence of ionomycin and BAPTA/AM which led to an elevation and decrease in intracellular calcium levels, respectively (Chou and Omary, 1991). This suggests that CAM kinases are less likely to be involved in K18 phosphorylation in interphase cells. Although our data suggest that PKCe and/or S6 kinase may phosphorylate K18 in vivo, other kinases could certainly be involved. For example, cAMP-dependent protein kinase, which was not examined in this study, may be involved since rat K8 and K18 can act as in vitro substrates for this kinase (Yano et al., 1991). However, 8-Br-cAMP had little effect on K8 and K18 phosphorylation in HT29 cells (Chou and Omary, 1991).

Several criteria support a potential role for a calcium-independent PKC such as PKCe in K8/18 phosphorylation (Omary et al., 1992): (a) the lack of phosphorylation of K18 by the calcium-dependent PKCa (Fig. 8), (b) the association of a PKCe related kinase with K8 and K18 as determined using biochemical means and double immunofluorescence labeling, (c) the decrease of basal K18 phosphorylation by introducing a pseudosubstrate of PKCe to intact cells by scratch loading, and (d) inhibition of in vitro phosphorylation of K8/18 immunoprecipitates by the associated kinase activity in the presence of PKCe pseudosubstrate. In addition, 23 of 37 identified PKC phosphorylation sites contained a hydrophobic residue in the +1 position of a phosphorylated serine (e.g., phe-53 of K18, Pearson and Kemp, 1991). Furthermore, the presence of two basic residues, particularly arginine, in the -3 and +2 positions is highly favorable for phosphorylation by PKC (e.g., arg-49 and 54 of K18, Kennedy and Krebs, 1991). Similar in vitro phosphorylation of rat liver K8/18 was obtained using PKC and CAM kinases (Yano et al., 1991).

Figure 9. Phosphorylation of K8/18 in NIH-3T3 cells expressing WT or ser-52→ala K18 mutant in the presence or absence of okadaic acid. Duplicate dishes of NIH-3T3 stable transfectant cells, expressing WT K8 and WT or ser-52→ala K18, were incubated with 10 mM sodium butyrate for 16 h to enhance keratin expression then labeled with 32PO4 for 4 h (250 μCi/ml). During the last 45 min of labeling, okadaic acid was added to one of the duplicate dishes followed by immunoprecipitation and SDS-PAGE analysis. Exposure was for 40 h (lanes a and b) and 2 h (lanes c and d). Nearly equivalent K8/18 portions were loaded from the wild-type and mutant immunoprecipitates.

Potential Kinases Involved in K18 Phosphorylation

The consensus sequence RXXXSXR for PKC, S6, and CAM kinases corresponds to the sequence *RSTSRFR containing ser-52 of K18. Our in vitro phosphorylation data showed that the ser-52 containing peptide was a major phosphopeptide that was phosphorylated by all three kinases (Fig. 6). In vivo phosphorylation of K8/18 in HT29 cells was previously shown to be calcium independent, based on unaltered K8/18 phosphorylation in the presence of ionomycin and BAPTA/AM which led to an elevation and decrease in intracellular calcium levels, respectively (Chou and Omary, 1991). This suggests that CAM kinases are less likely to be involved in K18 phosphorylation in interphase cells.

Although our data suggest that PKCe and/or S6 kinase may phosphorylate K18 in vivo, other kinases could certainly be involved. For example, cAMP-dependent protein kinase, which was not examined in this study, may be involved since rat K8 and K18 can act as in vitro substrates for this kinase (Yano et al., 1991). However, 8-Br-cAMP had little effect on K8 and K18 phosphorylation in HT29 cells (Chou and Omary, 1991).

Several criteria support a potential role for a calcium-independent PKC such as PKCe in K8/18 phosphorylation (Omary et al., 1992): (a) the lack of phosphorylation of K18 by the calcium-dependent PKCa (Fig. 8), (b) the association of a PKCe related kinase with K8 and K18 as determined using biochemical means and double immunofluorescence labeling, (c) the decrease of basal K18 phosphorylation by introducing a pseudosubstrate of PKCe to intact cells by scratch loading, and (d) inhibition of in vitro phosphorylation of K8/18 immunoprecipitates by the associated kinase activity in the presence of PKCe pseudosubstrate. In addition, 23 of 37 identified PKC phosphorylation sites contained a hydrophobic residue in the +1 position of a phosphorylated serine (e.g., phe-53 of K18, Pearson and Kemp, 1991). Furthermore, the presence of two basic residues, particularly arginine, in the -3 and +2 positions is highly favorable for phosphorylation by PKC (e.g., arg-49 and 54 of K18, Kennedy and Krebs, 1991). Similar in vitro phosphorylation of rat liver K8/18 was obtained using PKC and CAM kinases (Yano et al., 1991).
acid. The effect that we observed on disassembly was incomplete in that in the case of colcemid arrested cells for example, ~60% of the WT K18 transfected cells rearranged versus 30% in ser-52–ala K18 transfectants. Such an incomplete effect is not surprising, since phosphorylation of the K18 partner (i.e., K8) may be complementary or even dominant in having a disassembly/rearrangement role. Our working hypothesis is that phosphorylation at multiple sites that potentially involve both keratins will be important. Also, a simple on/off phosphorylation at a single site of a heteropolymeric protein pair may not necessarily occur, since multiple sites and multiple potential rearrangements with different functional consequences are likely to occur. For example, the double mutation at ser-22 and ser-392 of lamin A resulted in 56–73% of cells being unable to disassemble their nuclear lamina, whereas mutation of the ser-22 alone resulted in only 35% of the cells showing the mutant phenotype (Heald and McKeon, 1990). The only other study of the in vivo effect of altering phosphorylation sites on filament assembly and reorganization that we are aware of was done for ser-55 in vimentin, which is a site phosphorylated by p34cdc2 (Carpenter et al., 1992). In that study, a ser-55–ala mutation of vimentin resulted in normal assembly after transfection into SW13 cells lacking vimentin, but disassembly of the mutant protein did not occur during mitosis. In the case of K18, phosphorylation of the peptide containing ser-52 increases during mitotic arrest, but hyper- and neophosphorylation of four additional peptides also occurs (Chou and Omary, 1993). Edman degradation of the 32PO4-labeled ser-52 containing peptide (5STFSR) isolated from G2/M arrested cells showed that ser-52 is the only phosphorylated serine in that peptide (not shown).

Our identification of ser-52 as the major phosphorylation site of K18 in interphase cells, and its potential role in filament disassembly/rearrangement, offers an impetus and a model system for further characterization of the role of this and additional modification sites on filament rearrangement or interaction with other cellular elements. For example, phosphorylation of IF may play a role in their interaction with pectin (Foisner et al., 1991) or other associated proteins, in heatocyte response to epidermal growth factor (Baribault et al., 1989), or in islet cell insulin secretion (Schubert and Fields, 1984). As additional modification sites become characterized and with the availability of different modification mutants, a full picture should emerge in terms of the roles of K8/18 phosphorylation and glycosylation in filament dynamics.

We are grateful to Robert Oshima and Jerome Strauss for supplying K18 and K8 cDNA, respectively; Robert Evans for providing us with the SW13 cells; Howard Schulman, James L. Maller, and Berta Strolvici for their generous gift of CAM kinase, s6 kinase, and PKCc/z, respectively. We also thank Pierre Coulombe for stimulating discussions and his critical reading of the manuscript, and Linda Jacob for preparing the manuscript. This work was supported by a Veterans Administration Merit Award, National Institute on Alcohol Abuse and Alcoholism grant AA0947A-01, the PEW Scholars Program, and Digestive Disease Center grant DK38707.

Received for publication 6 April 1994 and in revised form 29 June 1994.

References


Ku and Omary Human Keratin 18 Phosphorylation 171