Abstract. Phosphorylation of keratin polypeptides 8 and 18 (K8/18) and other intermediate filament proteins results in their reorganization in vitro and in vivo. In order to study functional aspects of human K18 phosphorylation, we generated and purified a polyclonal antibody (termed 3055) that specifically recognizes a major phosphorylation site (ser52) of human K18 but not dephosphorylated K18 or a ser52→ala K18 mutant. Pulse-chase experiments followed by immunoprecipitation and peptide mapping of in vivo $^{32}$PO$_4^-$ labeled K8/18 indicated that the overall phosphorylation turnover rate is faster for K18 versus K8, and that ser52 of K18 is a highly dynamic phosphorylation site. Isoelectric focusing of $^{32}$PO$_4^-$ labeled K18 followed by immunoblotting with 3055 showed that the major phosphorylated K18 species contain ser52 phosphorylation but that some K18 molecules exist that are preferentially phosphorylated on K18 sites other than ser52. Immunoblotting of total cell lysates obtained from cells at different stages of the cell cycle showed that ser52 phosphorylation increases three to fourfold during the S and G2/M phases of the cell cycle. Immunofluorescence staining of cells at different stages of mitosis, using 3055 or other antibodies that recognize the total keratin pool, resulted in preferential binding of the 3055 antibody to the reorganized keratin fraction. Staining of human tissues or tissues from transgenic mice that express human K18 showed that the phospho-ser52 K18 species are located preferentially in the basolateral and apical domains in the liver and pancreas, respectively, but no preferential localization was noted in other simple epithelial organs examined. Our results support a model whereby phosphorylated intermediate filaments are localized in specific cellular domains depending on the tissue type and sites(s) of phosphorylation. In addition, ser52 of human K18 is a highly dynamic phosphorylation site that undergoes modulation during the S and G2/M phases of the cell cycle in association with filament reorganization.
Materials and Methods

Peptide Synthesis and Anti-phosphopeptide Antibody Generation

Peptide synthesis was carried out using an automated Milligen Peptide Synthesizer and FMOC-Cys Novasyn KA100 resin. The free hydroxyl group of serine was phosphorylated using dibenzyl-N-N-diethyl phosphoramidite as described (Ortvos et al., 1989; Andrews et al., 1991). The crude phosphopeptide was purified using reverse phase HPLC (3.9 × 300 mm C18 column) and a linear gradient containing 0.05% trifluoroacetic acid in 15-65% acetonitrile. The yield of the purified phosphopeptide was 50% (which elutes first) and purity of the HPLC purified product was confirmed using mass spectrometry (mass = 1367.92). The purified phosphopeptide 47VSRSTS(POg)FRGGMC or an identical nonphosphorylated peptide were coupled to keyhole limpet hemocyanin using MBS. Cys was included at the COOH terminus of the peptide to allow coupling to the carrier. Rabbits were immunized (intramuscularly, intradermally, and subcutaneously) at monthly intervals which provided high titer antibodies after four immunizations.

For antibody purification, the phosphorylated and nonphosphorylated peptides were reduced using the Reduce-Immunoblot Kit and coupled to SulfoLink™ columns (via the COOH-terminal cysteine residue) as recommended by the manufacturer. The peptide-coupled columns were then sequentially washed with 10 bed volumes of 10 mM Tris-HCl (pH 7.5), 100 mM glycine (pH 2.5), 10 mM Tris-HCl (pH 8.8), 100 mM triethylamine (pH 11), and 10 mM Tris-HCl (pH 7.5). Serum from the phosphopeptide immunized rabbits (4 ml diluted 1:10 in 10 mM Tris-HCl, pH 7.5) was passed over the nonphosphorylated-phosphate column three times to remove antibodies that bind to nonphosphorylated epitopes, then passed two times over the phosphorylated-peptide column. The column was washed with 20 bed volumes of 10 mM Tris-HCl (pH 7.5) and 10 mM Tris-HCl/500 mM NaCl (pH 7.5), followed by elution of the antibody using 10 bed volumes of 100 mM glycine (pH 2.5) with immediate neutralization using 5 ml of 1 M Tris-Cl (pH 8.0). The purified anti-phosphopeptide antibody (termmed 3055 Ab) was concentrated then dialyzed against PBS.

Cell Culture and Synchronization

HT29 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C (standard medium). Near-confluent cells were synchronized at the G1/S boundary by incubation with 5 μg/ml aphidicolin for 24 h. S phase and G2/M-enriched cells were then obtained by washing off the aphidicolin, splitting the cells 1:3, and then culturing in standard medium for different times. Cells were arrested at the G2/M boundary by treatment with colcemid (0.5 μg/ml, 36 h) as described (Chou and Omary, 1993). Heat stress of HT29 cells (at 50°C confluency) was carried out by culturing at 42°C for 24 h (Liao et al., 1995b). Cell cycle analysis was carried out as described (Chou and Omary, 1994).

Immunoprecipitation, Dephosphorylation, Western Blotting, and Gel Analysis

Cells were harvested, washed in PBS, and solubilized in PBS containing 1% NP-40 or 1% Empigen BB (Emp) and 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, 25 μg/ml aprotinin (4°C, 45 min), and 0.5 μg/ml okadaic acid. Insoluble material was pelleted and the detergent lysates were used for immunoprecipitation or Western blotting. Emp provides significantly higher yields of solubilized keratins than NP-40 (Lowthert et al., 1995). Detergent lysates (0.2–1.2 ml) were mixed with agarose-coupled L2A1 (20–40 μl) and incubated with shaking (2 h), washed 5× in the solubilization detergent, followed by SDS-PAGE (Laemmli, 1970).

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De-phosphorylation of K18, purified from asynchronous (G) or heat-stressed (42°C) cells by immunoprecipitation followed by electroelution of the K18 band, was carried out using 3 μg potato acid phosphatase (PAP) in 10 μl of 40 mM Pipes (pH 6.0) and 1 mM DTT for 1 h (22°C). Immunoblotting was done after separating lysates or immunoprecipitates using SDS-PAGE, and then transferring to PVDF membranes (4°C, 40 V, 12-16 h). Membranes were blocked in 5% nonfat dry milk/0.2% Tween 20 in PBS (MT) for 2 h at room temperature, and then incubated with the primary antibody diluted in MT (1:1,000 for CK5 or L2A1 ascites, 2 μg/ml for purified 30S5 antibody) for 1 h. After washing, membranes were incubated in a 1:1,000 dilution of peroxidase-conjugated goat anti-mouse IgG (for the L2A1, CK5 primary antibodies), or peroxidase-conjugated goat anti-rabbit IgG (for the 30S5 Ab) for 1 h, and then washed and visualized using an ECL system as recommended by the manufacturer. For the peptide competition experiments, Western blotting was carried out exactly as described except that the antibodies were preincubated with 150 μg/ml of nonphosphorylated or phosphorylated peptide followed by incubation with the blocked PVDF membrane. Two-dimensional gel electrophoresis was done using a gel apparatus (Mini-PROTEAN II; BioRad Laboratories, Cambridge, MA) exactly as recommended by the manufacturer.

**Figure 1.** Turnover of K8 and K18 phosphorylation. Subconfluent HT29 cells were labeled with 32P-O4 for 30 min (250 μCi/ml) in phosphate-free RPMI 1640 containing 10% dialyzed fetal bovine serum for 0.5 h, and then labeled for 30 min with 250 μCi/ml 32P-O4. After the pulse, the label-containing medium was replaced with normal RPMI 1640, and the cells were chased for 0, 1, 3, 4, or 6 h, followed by solubilization, immunoprecipitation, and SDS-PAGE analysis. Relative K8 and K18 protein levels were determined by scanning the wet destained gel using a Pharmacia-LKB Laser Densitometer. The gel was then dried and exposed to film followed by cutting out and counting K8, K18 and background-containing gel fragments to determine the specific activity of K8 and K19 labeling.

Tryptic phosphopeptide mapping was performed using keratins purified from cells (asynchronous [G cells]; G2/M colcemid arrested [M cells]; heat stressed [24 h, 42°C]; or cells treated with okadaic acid [OA]) that were labeled for 11 h with 250 μCi/ml 32P-O4 in the presence of 55 μM cold phosphate. Alternatively, cells were similarly labeled for 5 h (for the K18 ser52-containing peptide turnover experiment) then incubated in standard medium for 0, 3, or 6 h. Cells were solubilized followed by immunoprecipitation of K8/18. Tryptic peptide mapping was done as described (Boyle et al., 1991; Chou et al., 1993). Briefly, individual K8 and K18 were excised from preparative SDS-PAGE gels after brief Coomassie blue staining, and then electroeluted and aceton precipitated. The keratins were treated with trypsin (375 μg/ml in 80 μl of 50 mM NH4HCO3) for 16 h, lyophilized, and then electroelphoresed in the horizontal dimension, followed by chromatography in the vertical dimension. Where indicated, the relative radioactivity incorporated into individual phosphopeptides was determined by densitometric scanning of the peptide map autoradiographs. The stoichiometry of K8 and K18 phosphorylation in HT29 cells was determined using purified K8 and K18 that were separated by preparative SDS-PAGE followed by electroelution. Phosphate content was determined by ashing as described (Vacequier and Moy, 1991).

**Immunofluorescence**

HT29 cells were grown on coverslips, fixed in methanol (3 min, −20°C), washed once in PBS, followed by blocking with PBS containing 2.5% BSA (10 min). Tissues from mouse or human organs were frozen in O.C.T. compound (Miles, Elkhart, IN). Frozen sections were cut then fixed briefly in cold acetone. Fixed tissues or cells were then incubated with primary antibody (CK5 or L2A1 ascites, 1:100; or 3055 Ab, 10 μg/ml in the presence or absence of 200 μg/ml peptide or phosphopeptide) for 30 min. After washing in PBS/2.5% BSA, samples were blocked with 2% goat serum in PBS/2.5% BSA (15 min), followed by incubation with Texas red anti-rabbit (1:100; 3055 Ab) for 30 min. For DNA and keratin double staining, YO-PRO-1 iodide (Molecular Probes, Eugene, OR; 1:20,000) was included with the second antibody and all steps were carried out in the presence of RNase A (0.5 mg/ml). Slides were washed five times with PBS and photos were taken using Kodak Ektachrome color slide film from which black and white pictures were generated.

**Results**

**Turnover of K8 and K18 Phosphorylation**

Given that type I and type II keratins form obligate heteropolymers, we examined the turnover of phosphorylation at two levels: first, overall individual keratin phosphorylation and, second, specific phosphopeptide phosphorylation. Overall K8 and K18 phosphorylation was assessed by pulse labeling subconfluent human colonic HT29 cells with 32P-O4 for 30 min followed by chasing for different time points. As shown in Fig. 1, the turnover of K18 phosphorylation is significantly faster than that of K8. However, if confluent cells were used for labeling, the turnover differences between K8 and K18 phosphorylation became less dramatic (not shown). The initial increase in K8 phosphorylation after 1 h of chase is reproducible (n = 3) and suggests that the overall turnover of K8 phosphorylation is...
slower than that of K18, so that the net off/on rate at the early chase time point is more for K18 than for K8. Of note, the specific activity of radiolabeled K8 is approximately four times greater than for K18, although the overall stoichiometry of individual K8 and K18 phosphorylation after purification from confluent HT29 cells is 3.1 and 1.4 molecules of phosphate per molecule of K8 and K18, respectively.

Our previous studies indicated that ser52 is the major phosphorylation site of K18, after metabolic labeling of tissue cultured colonic tumor cell lines or normal human explant biopsies for 3 h with $^{32}$PO$_4$ (Ku and Omary, 1994). Other phosphopeptides become apparent as the duration of metabolic labeling is increased (not shown). This suggested that ser52 of K18 is likely to be a highly dynamic phosphorylation site. We addressed this possibility by labeling HT29 cells with $^{32}$PO$_4$ for 5 h then chasing for 0, 3, or 6 h in excess nonlabeled phosphate. As shown in Fig. 2A (d–f), ser52 shows significant relative dephosphorylation as compared with other K18 phosphopeptides. Using densitometric scanning, the phosphorylation ratio of ser52 phosphopeptide/two other K18 phosphopeptides is 1.5:1 at the 0 h chase versus 0.5:1 at the 6 h chase. The two major K8 peptides show slightly different turnover rates (Fig. 2A, a–c), with the peptide indicated by an arrowhead (Fig. 2A, a) having a 1.5× faster turnover rate than the second major K8 peptide. In addition, there are two faint K8 peptides that appear after the 6 h chase (Fig. 2A, indicated by arrows in c) which may represent slow turnover peptides or other more complex processes. Additional support for the rapid turnover of ser52 phosphorylation is the observation that when cells are labeled with $^{32}$PO$_4$ for 11 h in the absence of supplemental “cold” phosphate (i.e., under phosphate starvation conditions), ser52 shows minor radiolabeling as compared with other K18 phosphopeptides (not shown).

**Development of the 3055 Antibody Directed to the K18 Phospho-ser52-containing Epitope**

Given that ser52 of K18 represents a highly dynamic phosphorylation site, we proceeded to develop antibodies that specifically recognize this phospho-epitope to address functional questions regarding this phosphorylation site. Specificity of the generated antibodies (term ed 3055 Ab) was confirmed at several levels.

First, immunoblotting of wild type (WT) and ser52→ala K18, both expressed then purified from Sf9 cells (Ku and Omary, 1994), using purified 3055 Ab showed that the antibody reactivity with WT K18 was abolished in the case of the ser52 mutant (Fig. 3A, compare lanes 5 and 6). The 3055 Ab reactivity is specifically abolished in the presence of the phosphopeptide used for immunization (Fig. 3A, lanes 7 and 8) but is not affected by the presence of peptide alone (Fig. 3A, lanes 5 and 6). In all cases, equal amounts of protein were analyzed as determined by Coomassie staining (Fig. 3A, lanes 1 and 2) and immunoblotting using antibodies that recognize the total K18 pool (Fig. 3A, lanes 3 and 4 for CK5 antibody, and not shown for L2A1 antibody).

Secondly, we used the 3055 Ab to examine K18 ser52 phosphorylation under conditions that previously showed overall K18 hyperphosphorylation. These include mitotic arrest (Chou and Omary, 1993), heat stress (Liao et al., 1995b), and treatment with the phosphatase inhibitor okadaic acid (Ku and Omary, 1994). As shown in Fig. 3B, a dramatic increase in K18 ser52 phosphorylation was noted.

![Figure 2](image-url)

**Figure 2.** Turnover of K8 and K18 phosphopeptides and K18 tryptic phosphopeptide mapping. (A) HT29 cells were labeled for 5 h with $^{32}$PO$_4$ then chased for 0, 3, or 6 h in normal medium as described in Materials and Methods. Individual K8 and K18 bands were then purified followed by tryptic peptide mapping. Small x indicates the origin where samples were loaded on the TLC plates. Arrowhead in a indicates higher turnover K8 phosphopeptide, and S52(d–f) indicates the ser52 phosphopeptide. For each map, 1,000 cpm were spotted, and the overall specific activity of radiolabeled K8 and K18 decreased progressively after the 3 then 6 h chase (not shown). (B) K18 was isolated from HT29 cells that were grown under four conditions: asynchronous growth (G), heat stress (42°C, 24 h), G cells treated with okadaic acid (OA), or cells arrested at G2/M using colcemid (M) as described in Materials and Methods. Cells were labeled with $^{32}$PO$_4$ for the last 11 h of each condition. For OA treatment, the phosphatase inhibitor was added during the last 2 h of labeling. Arrows indicate the ser52 phosphopeptide.
Characterization of antibodies to the phospho-ser52 containing epitope. (A) The 3055 Ab was generated and purified as described in Materials and Methods. Equal amounts of WT K18 or ser52→ala K18 (S*), expressed then purified from insect Sf9 cells, were analyzed by immunoblotting using mAb CK5 (which recognizes phosphorylated and nonphosphorylated K18) and 3055 antibody in the presence of excess phosphorylated or nonphosphorylated peptide containing ser52. (B) SDS detergent lysates (10 μg) from asynchronously growing cells (G), cells arrested at the G2/M stage of the cell cycle using colcemid (M), heat stressed cells (42°C), or cells treated with OA for 2 h were separated by SDS-PAGE (lanes 1-4), transferred to a PVDF membrane then analyzed by immunoblotting using antibody 3055 (lanes 5-8) or antibodies CK5 and L2A1. The Coomassie stain shown was done on the same PVDF membrane, after completion of the immunoblotting. (C) K18 was purified from G or heat stressed cells. Equivalent amounts of purified K18 were incubated alone or in the presence of potato acid phosphatase (PAP), and then analyzed by immunoblotting using CK5 or 3055 antibodies.

After immunoblotting using the 3055 Ab in heat stressed, OA-treated and M cells as compared with G cells, while the amount of K18 analyzed was nearly equal as determined by immunoblotting using L2A1 and CK5 antibodies. The purified 3055 Ab is highly specific and recognizes only the ser52 phosphorylated K18 among the large pool of proteins in the solubilized cellular protein lysate (Fig. 3 B). Tryptic phosphopeptide mapping of K18 isolated from 32P04-labeled G, M, 42°C and OA-treated cells all showed that S52 is a labeled phosphopeptide (Fig. 2 B), albeit the overall pattern of K18 tryptic phosphopeptides for each condition differed somewhat. Although ser52 phosphorylation increased during heat stress (Fig. 3 B), the relative increase is not as high as the increase in other phosphopeptides since tryptic phosphopeptide mapping (Fig. 2 B) shows that the ser52-containing phosphopeptide is not the major labeled phosphopeptide (compare b in Fig. 2 B with a, c, and d).

Third, to further validate that it is in fact the phosphate on ser52 that is important for 3055 Ab reactivity, K18 was isolated from G and 42°C stressed cells followed by potato acid phosphatase treatment which abolished 3055 Ab reactivity (Fig. 3 C, compare lanes a and b with c and d). Specificity of the 3055 antibody was also examined by immunofluorescence staining of HT29 cells cultured at 37°C or 42°C. As shown in Fig. 4, reactivity with the 3055 Ab under both conditions was abolished in the presence of the phosphopeptide (c and g with insets showing nuclear double staining of the corresponding panels) but not in the presence of the peptide (d and h). Neither the peptide nor the phosphopeptide have any effect on the immunofluorescence staining pattern obtained using L2A1 on CK5 antibodies (not shown). As we reported previously (Liao et al., 1995b), heat stress results in significant reorganization of K8/18 filaments as exemplified by f and h in Fig. 4. Double staining with 3055 and L2A1 (or CK5) antibodies showed that the 3055 Ab preferentially recognized reorganized filaments of heat stressed cells (not shown but see Fig. 7). Although the 3055 Ab is monospecific with regard to its binding to phospho-ser52 of K18, it also does react (by immunoblotting) with a 66-kD epidermal keratin only after incubation of cultured keratinocytes with okadaic acid (not shown).

Isoelectric Focusing/SDS-PAGE Analysis of Keratin Phosphorylation

We examined the phosphorylated isoforms of K18 by comparing the in vivo labeled species with species that are recognized by the 3055 Ab. In vivo 32PO4 labeling of K18 results in one major and three minor phosphorylated species, whereas 3055 Ab immunoblotting shows two major and two minor ser52 phosphorylated K18 species (Fig. 5). This analysis indicates that a significant fraction of phosphorylated K18 has ser52 phosphorylation but that other minor phosphorylated K18 populations occur which are either preferentially phosphorylated at sites other than ser52 (e.g. fourth K18 Coomassie stained spot from left in Fig. 5 B) or that are phosphorylated on ser52 as well as on other sites (see schematic in Fig. 5 B). Similar to K18, analysis of the overall K8 and K19 phosphorylation (K8/18/19 coimmunoprecipitate) showed that the major protein fractions of K8 and K19 are not phosphorylated (Fig. 5). We do not know the nature of the difference between the two non-phosphorylated forms of K18 shown in Fig. 5 B. The more
acidic nonlabeled isoform is noted particularly after Emp solubilization and is not usually seen after NP-40 solubilization (not shown). It could represent a hitherto undefined charged modification (e.g., sulfation) or a very slow turnover non-ser52 phosphorylation site.

Analysis of K18 ser52 Phosphorylation during Cell Cycle Progression

We previously showed that K8/18 overall phosphorylation increases during the S and G2/M stages of the cell cycle in cells synchronized by G1/S aphidicolin block followed by release of the block (Chou and Omary, 1994). We tested K18 ser52 phosphorylation during different stages of the cell cycle by immunoblotting cell lysates with the 3055 Ab. As shown in Fig. 6, the peak of cells at the S and G2/M phases of the cell cycle was after 7 and 12 h after removing the aphidicolin, respectively, and corresponded to a threefold (S phase enriched cells, lane 3) and fourfold (G2/M phase enriched cells, lane 4) increase in ser52 phosphorylation. The increase in ser52 phosphorylation is reversible as cells exit from the G2/M stage (Fig. 6, lanes 5 and 6).

We then compared the arrangement of K8/18 filaments during different stages of mitosis, as determined by immunostaining using L2A1 or 3055 antibodies. As shown in Fig. 7 A, staining of mitotic cells using the 3055 Ab was much brighter than the background nonmitotic cell staining which was a reflection of the observed increase in ser52 phosphorylation of K18 (Fig. 6). In addition, the 3055 Ab preferentially recognized the reorganized filaments which cannot be appreciated using mAb CK5 (not shown) and mAb L2A1 (Fig. 7 A) which recognize the total keratin pool. Preferential binding to reorganized filaments was also confirmed by double staining using L2A1 and 3055 antibodies (Fig. 7 B). Omission of the 3055 and L2A1 antibodies provided blank staining similar to that shown in c and g of Fig. 4 (not shown).

Polarity of Phospho-ser52 K18 in Tissues

We tested if a unique distribution of the phosphorylated K18 fraction can be identified in tissues, as compared to the overall phosphorylated and nonphosphorylated K18 pools. As shown in Fig. 8 (b and d), staining of human liver and pancreas with the 3055 Ab showed preferential localization to the basolateral and apical membrane domains, respectively. This contrasts with the membrane and cytoplasmic staining observed with the L2A1 antibody (Fig. 8, a and c) and CK5 antibody (not shown). Similar results were observed using transgenic mice that overexpress normal human K18 (Fig. 8, i–l). Interestingly, this polarity of phospho-K18 distribution was not observed in all tissues. For example, human kidney and colon did not show the polarity of distribution of the phospho-ser52 K18 (Fig. 8, e–h), which was also the case in transgenic mouse kidney and intestinal tissues (not shown). The immunofluorescence results shown in Fig. 8 do not take into account the potential presence of any soluble phospho-ser52-K18 which would provide a diffuse staining pattern. We were not able to make use of the antibody to the nonphosphorylated

Figure 4. Immunofluorescence of HT29 cells using L2A1 and 3055 antibodies. HT29 cells were cultured at 37° or 42°C (24 h) on cover slips, rinsed, and then fixed with 100% methanol for 3 min (−20°C). Fixed cells were incubated with mAb L2A1 or with 3055 Ab (10 μg/ml) alone or in the presence of excess nonphosphorylated or phosphorylated peptide (200 μg/ml). After 30 min, cells were rinsed followed by incubating with RNase A and the DNA staining dye YO-PRO1, and Texas red conjugated goat anti-mouse or goat anti-rabbit antibodies as described in Materials and Methods. Insets in c and g reflect nuclear double staining.
Figure 5. Two dimensional gel analysis of keratin phosphorylation. (A) Asynchronously growing HT29 cells were labeled with $^{32}$PO$_4$ for 11 h followed by immunoprecipitation of K8/18/19 using mAb L2A1. Immunoprecipitates were analyzed by isoelectric focusing (IEF) in the horizontal direction then SDS-PAGE in the vertical direction. The gel was then transferred to a PVDF membrane followed by Coomassie staining then autoradiography of the same membrane. (B) Given that the same membrane (A) was used for the analyses shown, a qualitative schematic was generated to show the relative Coomassie intensity, $^{32}$PO$_4$ incorporation, and 3055 Ab reactivity of K8, K18 and K19. Bold squares, relative Coomassie staining intensity; +, relative $^{32}$PO$_4$ incorporation; *, relative 3055 immunoblot intensity.

ser52-containing peptide that we generated since despite using two rabbits and purification of the antibody with a peptide conjugated column, it still recognized K18 plus two other major crossreacting bands (not shown).

Figure 6. Phosphorylation of K18 ser52 increases during the S and G2/M phases of the cell cycle. Asynchronously growing HT29 cells (lane 1) were cultured in the presence of aphidicolin (5 μg/ml) for 24 h which results in cell block at G1/S. Cells were then split 1:3 and cultured in the absence of aphidicolin for the indicated times. At each time point, one dish was used for cell cycle analysis and a duplicate dish was used for immunoblotting with CK5 or 3055 antibodies. Equal amounts of protein (10 μg) were analyzed for each lane. The increase in ser52 phosphorylation level was determined by comparing the densitometric scanning of lanes 2–4 with a standard curve of increasing concentration of cell lysate separated on the same gel (not shown).

Discussion

Dynamics of K8/18 Phosphorylation

Of the cytoplasmic IF proteins, keratins and neurofilaments are unique in that they form obligate heteropolymers. The neurofilament heteropolymers consist of the light (L), middle (M), and heavy (H) chains. Both type I and type II keratin chains are phosphorylated with type II being generally more heavily phosphorylated than type I keratins (e.g., Yano et al., 1991b; Chou and Omary, 1993), and similarly, all NF chains are phosphorylated with NF-H having the highest stoichiometry of phosphorylation (Julien and Mushynski, 1983). Several phosphorylation-related features are shared between NF and K8/18. For example, phosphorylation is preferentially localized to the head domain of K18 (Chou and Omary, 1993), or the head and tail domains in NF-L, M, H (reviewed by Nixon and Sihag, 1991), and K8 (Chou and Omary, 1993; and unpublished observations). These are the domains that impart most of the structural heterogeneity among IF proteins and hence are likely to be involved in regulating their tissue specific function(s) (reviewed in Steinert and Roop, 1988; Fuchs and Weber, 1994). In addition, the turnover of
phosphorylation of NF varies among the individual NF-L, M, and H chains as does the phosphorylation turnover between K8 and K18. For example, the turnover of phosphorylation in NF-L and NF-M is significantly faster than that in NF-H (Sihag and Nixon, 1991). With regard to K8 and K18, although the stoichiometry of phosphorylation is K8 > K18 (Fig. 5), the percent turnover of phosphorylation is K18 > K8 (Fig. 1). This suggests that phosphorylation of K8 may not be always intimately tied with phosphorylation of K18, and that modification of each keratin is likely to play separate functions. To that end, we previously observed that mutation of ser52 of K18 does not affect the overall phosphorylation of K8 or the individual labeled phosphopeptides (Ku and Omary, 1994). Furthermore, although phosphorylation of K8 and K18 increased by similar proportion under conditions such as heat stress (Liao et al., 1995b), G2/M arrest (Chou and Omary, 1993), and progression through the cell cycle (Chou and Omary, 1994), there are several examples where alteration of the type II keratin (e.g., K8) appears to occur preferentially. These include hyperphosphorylation of the type II keratin upon stimulation of cultured rat hepatocytes with epidermal growth factor (Baribault et al., 1989), stimulation of human amniotic cells by pro-urokinase (Busso et al., 1994), or during meiotic maturation in the *Xenopus* oocyte (Klymkowsky et al., 1991). However, depending on the efficiency of radiolabeling, it may be difficult to appreciate a change in K18 phosphorylation under conditions where immunopurification of K8/18 is not done, since incorporation of $^{32}$PO$_4$ into K18 is limited as compared with K8.

**Use of Anti-phospho-epitope Antibodies to Study Protein Phosphorylation**

The purified 3055 Ab described in this study is highly specific to the phospho-ser52 epitope (Figs. 3–5). Purification of the antibody was essential since the nonpurified 3055 Ab cross-reacted with two other unknown proteins (not shown). Although ser52 is a major phosphorylation site of K18, several other phosphopeptides are noted after labeling to equilibrium with $^{32}$PO$_4$ (Fig. 2 A). Immunoblotting of isoelectrically separated K18 isoforms that were pre-labeled with $^{32}$PO$_4$ showed that all the isoforms contained ser52 phosphorylation but that one isoform had preferential phosphorylation at other site(s) (Fig. 5).

Several studies described the use of antibodies targeted to serine-containing specific phospho-epitopes (reviewed by Czernik et al., 1991). These include the generation of monoclonal (Yano et al., 1991a) and polyclonal antibodies (Nishizawa et al., 1991; Matsuoka et al., 1992) targeted to GFAP phospho-epitopes, and antibodies to the phospho-KSPV motif in tau protein (Lee et al., 1991), and to vimentin phospho-epitope (Tsujimura et al., 1994). The epitopes for these antibodies were predesigned and well defined in contrast to antibodies that were generated to phosphorylated NF whose epitopes were not known. Examples of the latter include antibodies that recognize phospho-NF-L where the immunogen was a hypothalamic tissue homogenate (Sternberger and Sternberger, 1983), or antibodies that recognize NF-M, H where the immunogen was purified NF (Lee et al., 1987). Antibodies that specifically recognize phosphotyrosine containing epitopes have also been described which include an epitope on c-erb (Epstein at al., 1992; Bangalore et al., 1992; DiGiovanna and Stern, 1995) or an epitope to β-integrin (Jo hannson et al., 1994). In general, these phosphoserine and phosphotyrosine-containing epitope tailored antibodies are extremely specific reagents that recognize only the phosphorylated residue in the context of an epitope (e.g., Figs. 3 and 4), and are blocked by the phosphopeptide used for immunization but not by the phosphoamino acid. Such phospho-epitope specific antibodies can even distinguish between two adjacent phosphorylated serine or threonine as demonstrated for polyclonal antibodies to the epitopes RAST*I ... and ... RAS*TI ... of phospholamban (Drago and Colyer, 1994).

**Dynamics and Significance of K18 ser52 Phosphorylation**

Several observations indicate that ser52 of K18 is a highly dynamic phosphorylation site. First, short-term labeling (3 h) of tissue cultured cells or normal colonic biopsies with $^{32}$PO$_4$ results in 80 or 95% of the K18 radiolabeled counts being incorporated into ser52, respectively (Ku and Omary, 1994). Second, ser52 phosphorylation increases three-
Figure 8. Polarity of phosphorylated keratin distribution in human and mouse tissues. Normal human liver, pancreas, colon and kidney, or liver and pancreas tissues from transgenic mice expressing WT human K18 were used as described in Materials and Methods. Tissues were snap frozen in O.C.T., sectioned, and then stained with mAb L2A1 or with the 3055 Ab (30 min). After washing, sections were incubated with Texas red–conjugated goat anti-mouse or anti–rabbit antibodies. Similar staining to that with L2A1 was observed when mAb CK5 was used (not shown).

fourfold during the S and G2/M phase of the cell cycle in a reversible manner (Fig. 6), with subsequent decrease in phosphorylation as cells exit G2/M and enter G1. One likely function for this increase in phosphorylation is a role in the reorganization of the keratin filaments. In support of this, ser52→ala mutation interferes with the ability of K8/18 filaments to reorganize in transfected cells arrested in G2/M or treated with OA (Ku and Omary, 1994). Furthermore, the 3055 Ab preferentially recognizes reorganized filaments during mitosis (Fig. 7) thereby further supporting a role for K18 ser52 phosphorylation in filament reorganization. The contribution to filament reorganization of other K18 or K8 phosphorylation sites remains to be determined as these sites become identified and studied. Third, peptide mapping of pulse-chased K18, that is metabolically labeled with $^{32}$PO$_4$, shows that ser52 of K18 is a rapid turnover phosphorylation site (Fig. 2 B).

Our results support several potential functions for K18 ser52 phosphorylation. One function is filament reorganization as mentioned above. A second function is to dictate specific localization domains of K8/18 in polarized cells of the pancreas and liver. Such a potential function will still need additional supportive data. However, the preferential localization of phospho-ser52-K18 in the basolateral domain of hepatocytes and apical domain of pancreatic cells suggests that the phospho-ser52-K18 fraction interacts with specific cellular elements that are localized in these domains, and that the phosphorylation of ser52 is important for that interaction. The nature of such putative domain-specific K8/18 interacting elements remains to be determined (see below). Specificity of this localization is supported by its lack of distribution in all tissues. It is not clear at this stage why a polar distribution of phospho-ser52 K18 was found in the liver and pancreas but not in the kidney or colon (Fig. 8). It is possible that phosphorylation of sites other than ser52 of K18 (on K8 or K18) would impart a polar distribution of the phosphorylated species in the colon and kidney that is similar to what we
observed for phospho-ser52 K18 in the liver or pancreas. Alternatively, the “elements” that interact with ser52-phosphorylated K18 may not be present or are not polarized in the colon and kidney under the conditions examined. A third function is to alter K8/18 solubility which in turn increases the level of cytoplasmic nonfilamentous, nonmembrane-associated K8/18 that may interact with other soluble proteins such as heat shock protein 70 (Liao et al., 1995a). Although we do not have direct evidence that specific phosphorylation at ser52 of K18 affects solubility of K8/18, several examples of concurrent hyperphosphorylation of K8 and K18 (with increased phosphorylation at ser52; Fig. 3B) correlate with increased K8/18 solubility. These examples include heat stress (Liao et al., 1995a,b), colcemid arrest at G2/M (Chou et al., 1993), and treatment with okadaic acid (not shown).

**Potential Implications for the Polarity of Distribution of Phosphorylated Cytoplasmic IF Proteins**

Epithelial cells and neurons have the common feature of polar cellular domains whereby some neuronal proteins found in the axon differ from those found in the cell body and dendrites and, similarly, epithelial proteins found in the apical domain differ from those found in the basolateral domain (reviewed by Rodriguez-Boulan and Powell, 1992; Matter and Mellman, 1994). The polar distribution of phospho-ser52 K18 in some epithelial tissues (Fig. 8) is reminiscent of the polar distribution of phosphorylated NF in neurons (Sternberger and Sternberger, 1983; Lee et al., 1987). This suggests that other cytoplasmic IF proteins may manifest a similar polarity distribution of their phosphorylated fraction. Such a hypothesis is attractive but will require additional testing in several systems. To that end, the restricted spatial distribution of phosphorylated keratins and NF in nondividing tissues parallels the spatiotemporal distribution of phospho-thr7, ser13, ser34-GFAP at the cleavage furrow during cytokinesis in cultured astroglial cells (reviewed by Inagaki et al., 1994).

The polarized staining pattern of liver and pancreatic tissues with the 3055 Ab showed a continuous nonpunctate pattern in proximity to the plasma membrane (Fig. 8). Several candidate proteins that may be involved in the interaction with phospho-ser52 K18 include desmoplakin (DP), fodrin, or other cell surface associated proteins such as integrins. Although to our knowledge a fodrin-keratin interaction has not been described, such an interaction would not be surprising given the polarized distribution of fodrin in epithelial cells (reviewed by Bennett and Gilligan, 1993), and the described association between fodrin and other IF proteins including vimentin (Mangeat and Burridge, 1984), NF (Frappier et al., 1987; Langley and Cohen, 1987), and peripherin (Takeamura et al., 1993). In addition, cell surface associated receptors could also potentially interact with the phospho-ser52 K18. For example, a high mannose and complex oligosaccharide-containing 85-kD glycoprotein, that is likely to be a cell surface protein, associated with K8/18 (Chou et al., 1994). Also, different integrins show a polarized distribution in epithelial cells (De Luca et al., 1990), with potential association of the integrin β4 subunit with the keratin fraction in mouse carcinoma cell lines (Gomez et al., 1992). With regard to DP, its association with keratins has been studied in several systems (e.g., Stappenbeck et al., 1993). To that end, a unique domain in several type II keratins was found to be important for binding to DP although this domain is not found in vimentin or K8/18 which are also known to bind to DP (Kouklis et al., 1994). It is not known if phosphorylation of K8/18 regulates their interaction with DP although phosphorylation of a serine in the COOH-terminal domain of DP interferes with its interaction with K8/18 (Stappenbeck et al., 1994). Any potential role for K8 ser52 phosphorylation in K8/18-DP interaction remains to be tested, but the known punctate staining pattern of DP (Pasdar and Nelson, 1988) suggests that the linear staining pattern of phospho-ser52 K18 cannot be exclusively accounted for by a phospho K18-DP interaction.

Although cell polarity has been extensively studied in epithelial cells, all cells manifest some degree of polarity or asymmetry (Matter and Mellman, 1994). The use of anti-phosphoepitope antibodies directed to IF proteins should provide important functional information based on specific polarity or spatiotemporal cellular distribution, and also provide a handle for studying the regulation of IF proteins.

We are very grateful to Dr. Robert Oshima for providing us with the transgenic mouse that express human keratin 18, Dr. Masaki Inagaki for advice regarding generation of the 3055 antibody, Kris Morrow for constructing the figures, Linda Jacob and Sally Morefield for preparing the manuscript, Evelyn Resurreccion for performing the tissue sectioning and staining, Dr. Warren Hoeffler for providing us with cultured human keratinocytes, and Lusijah Rott for assistance with cell cycle analysis.

This work was supported by a Department of Veterans Affairs Merit Award, National Institutes of Health grants AA0947A-01 and DK 47918, and Digestive Disease Center Grant DK38707. N.-O. Ku is a recipient of an American Heart Association California Affiliate postdoctoral fellowship.

Received for publication 25 July 1995 and in revised form 11 September 1995.

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