Phosphorylation of human keratin 18 serine 33 regulates binding to 14-3-3 proteins

Nam-On Ku¹, Jian Liao² and M.Bishr Omary³

VA Palo Alto Health Care System, 3801 Miranda Avenue, 154J, Palo Alto, CA 94304 and the Digestive Disease Center, Stanford University School of Medicine, Stanford, USA

²Present address: Clontech Laboratories Inc., Palo Alto, CA, USA

³Corresponding author

¹Author for reprint requests

N.-O.Ku and J.Liao contributed equally to this work

Members of the 14-3-3 protein family bind the human intermediate filament protein keratin 18 (K18) *in vivo*, in a cell-cycle- and phosphorylation-dependent manner. We identified K18 Ser33 as an interphase phosphorylation site, which increases its phosphorylation during mitosis in cultured cells and regenerating liver, and as an *in vitro* cdc2 kinase phosphorylation site. Comparison of wild-type versus K18 Ser33→Ala/Asp transfected cells showed that K18 Ser33 phosphorylation is essential for the association of K18 with 14-3-3 proteins, and plays a role in keratin organization and distribution. Mutation of another K18 major phosphorylation site (Ser52) or K18 glycosylation sites had no effect on the binding of K18 to 14-3-3 proteins. The K18 phospho-Ser33 motif is different from several 14-3-3-binding phosphomotifs already described. Antibodies that are specific to K18 phospho-Ser33 or phospho-Ser52 show that although Ser52 and Ser33 phosphorylated K18 molecules manifest partial colocalization, these phosphorylation events reside predominantly on distinct K18 molecules. Our results demonstrate a unique K18 phosphorylation site that is necessary but not sufficient for K18 binding to 14-3-3 proteins. This binding is likely to involve one or more mitotic events coupled to K18 Ser33 phosphorylation, and plays a role in keratin subcellular distribution. Physiological Ser52 or Ser33 phosphorylation on distinct K18 molecules suggests functional compartmentalization of these modifications.

Keywords: keratin 18/keratin phosphorylation/14-3-3 proteins

Introduction

Intermediate filament (IF) proteins consist of a large family of nuclear (i.e. lamins) and cytoplasmic [e.g. keratins (K), neurofilaments, vimentin, desmin] cytoskeletal proteins (reviewed by Steinert and Roop, 1988; Fuchs and Weber, 1994). Cytoplasmic IF protein expression is tissue specific as exemplified by keratins in epithelial cells, vimentin in mesenchymal cells, desmin in muscle and neurofilaments in neuronal cells. All IF proteins have three characteristic and common structural domains: a central α -helical 'rod'

domain of 310–350 amino acids which is flanked by non- α -helical N-terminal 'head' and C-terminal 'tail' domains. Most of the structural diversity in IF proteins occurs at the head and tail domains, which are the domains that contain the glycosylation and phosphorylation sites of these proteins (reviewed by Ku *et al.*, 1996).

The largest subfamily of IF proteins, namely the keratins (Moll *et al.*, 1982) (numbered K1–K20), have several important biological and clinically relevant features. As such, epithelial tissues express at least one type I (i.e. K9–K20) and one type II (i.e. K1–K8) keratin in a cell-specific manner. For example, simple-type epithelia (e.g. liver, intestine and exocrine pancreas) express K8/18, and keratinocytes express K5/14 in the basal proliferative compartment or K1/10 in the suprabasal differentiated compartment. Mutations in 13 of the >20 known keratins result in several oral or skin diseases (McLean and Lane, 1995; Fuchs 1996; Irvine *et al.*, 1997). In addition, several animal models, as well as a K18 mutation that was identified in a patient with cryptogenic cirrhosis (Ku *et al.*, 1997), suggest that K18 mutations may cause or result in a predisposition to human liver disease (reviewed by Omary and Ku, 1997).

Although the function(s) of cytoplasmic IF proteins, including K8/18, are poorly understood, it is likely that IF are similar to other proteins in that at least some aspects of their function are regulated by their associated proteins and post-translational modifications. In the case of K8/18, both modes of regulation are highly dynamic (Omary *et al.*, 1998). For example, K18 Ser52 phosphorylation undergoes rapid turnover, is distributed in a preferentially polarized fashion in tissues and plays a role in filament reorganization (Ku and Omary 1994; Liao *et al.*, 1995a). With regard to keratin-associated proteins, several members of the 14-3-3 protein family associate reversibly with K8/18 in cultured cells and human colonic biopsies, and in stoichiometrically significant, cell-cycle-regulated and phosphorylation-dependent fashions (Liao and Omary, 1996). 14-3-3 proteins were first described in the brain (Moore and Perez, 1967) and include several members (e.g. β , ζ , γ , τ , ϵ , η) that range in size between 28 and 33 kDa (reviewed by Aitken, 1995, 1996). The α and δ 14-3-3 proteins represent the phosphorylated forms of β and ζ 14-3-3 proteins, respectively (Aitken *et al.*, 1995). 14-3-3 proteins bind to several functionally distinct molecules including neurotransmitter biosynthetic enzymes (Ichimura *et al.*, 1988; Furukawa *et al.*, 1993), kinases (Freed *et al.*, 1994; Fu *et al.*, 1994; Reuther *et al.*, 1994; Bonnefoy-Berard *et al.*, 1995; Braselmann and McCormick, 1995), phosphatases (Conklin *et al.*, 1995), and oncogene products including middle T antigen (Pallas *et al.*, 1994) and Cbl (Liu *et al.*, 1996), and as such, one of the proposed functions of 14-3-3 proteins is to regulate the enzyme activity of its binding proteins. The association

of 14-3-3 proteins with phosphorylated K8/18 occurs in the cytosolic and membrane-associated compartments during the S and G₂/M phases of the cell cycle (Liao and Omary, 1996).

In cases that have been examined, binding of 14-3-3 to an associated protein is dependent on phosphorylation of the associated protein. This includes binding of 14-3-3 to Raf and Bcr kinases (Michaud *et al.*, 1995), tryptophan hydroxylase (Furukawa *et al.*, 1993; Banik *et al.*, 1997), the Bcl-2 family member 'death agonist' BAD (Zha *et al.*, 1996), cdc25 (Peng *et al.*, 1997), protein tyrosine phosphatase PTPH1 (Zhang *et al.*, 1997) and K18 (Liao and Omary, 1996). The 14-3-3 binding motif RXXS(PO₄)XP, which includes Ser259 and Ser621 of Raf-1, was proposed as a general 14-3-3 binding motif (Muslin *et al.*, 1996). This motif is present in several 14-3-3 binding proteins (e.g. B-Raf, polyoma middle T antigen, protein kinase C, tyrosine hydroxylase), but it is not known if the corresponding motif in these proteins is indeed responsible for binding of 14-3-3 proteins. Phosphorylation at Ser112 or Ser136 of BAD (Zha *et al.*, 1996), or at Ser216 of cdc25 (Peng *et al.*, 1997), which share an RXXSXP sequence, was shown to be essential for binding to 14-3-3 proteins. In addition, a related phospho-motif (RXXS) in PTPH1 also plays an important role in binding of 14-3-3 proteins (Zhang *et al.*, 1997). However, the 14-3-3 binding protein Cbl does not contain an RXXSXP motif, and double mutation of two serines at two different serine-rich regions of Cbl abrogated binding of 14-3-3 proteins (Liu *et al.*, 1997). Although phosphorylation of the mutated Cbl serines was not demonstrated, studies with the Cbl proto-oncogene suggest that 14-3-3 proteins could bind more than one phosphorylated sequence motif.

In this report, we identified K18 phospho-Ser33 as the site that regulates keratin/14-3-3 binding during mitosis, and showed that K18 Ser33 phosphorylation generates a unique motif that is essential but not sufficient for binding of 14-3-3 proteins. K18 Ser33→Ala/Asp mutants inhibit binding of 14-3-3 proteins and alter keratin organization and distribution. Our data provides insight into the functional significance of association of keratin with 14-3-3 proteins.

Results

Mitotic phosphorylation of K18 Ser33 correlates with binding of 14-3-3 proteins to K8/18

Our previous results showed that K18 association with 14-3-3 proteins occurs in a K18 phosphorylation- and cell-cycle-dependent manner (Liao and Omary, 1996). Here, we used two systems of G₂/M enriched cells that included cell synchronization with the DNA polymerase α inhibitor aphidicolin or by arresting cells using colcemid. As shown in Figure 1A, both methods are associated with a significant, Coomassie-stain detectable, association of 14-3-3 proteins with K8/18 immunoprecipitates. This was confirmed by blotting with an antibody that recognizes several 14-3-3 isoforms (Figure 1A). In order to identify the K18 phosphorylation site(s) that regulate binding to 14-3-3 proteins, we investigated whether a unique *in vivo* ³²PO₄-labeled spot in the K18 phosphopeptide map increased during the G₂/M phase of the cell cycle. As

Fig. 1. Association of 14-3-3 proteins with keratins and tryptic peptide mapping of ³²PO₄-labeled K18 isolated from asynchronous or G₂/M-enriched cells. G₂/M-enriched HT29 cells were obtained using: (i) cell synchronization with aphidicolin (5 μ g/ml, 24 h), followed by washing off the drug and allowing cells to proceed through the cell cycle for 10 h or (ii) arrest with colcemid (5 μ g/ml) for 36 h. Near confluent non-drug-treated asynchronously growing cells were used as G₀/G₁ enriched cells. (A) K8/18 precipitates were obtained from the indicated cells using mAb L2A1, analyzed by SDS-PAGE, then stained with Coomassie Blue. Duplicate precipitates were blotted with anti-14-3-3 Ab. The small arrows pointing to the faint bands below and above the K8 band correspond to K20 and a hyperphosphorylated form of K8, respectively (Liao *et al.*, 1997). (B) HT29 cells were labeled for the last 5 h of each condition (250 μ Ci/ml of ³²PO₄) followed by precipitation of K8/18, then separation of K8 from K18 using preparative SDS-PAGE. Duplicate dishes, under the same labeling conditions but without added radioactivity, were used for cell cycle analysis with % G₀/G₁, S and G₂/M indicated for each cell condition. Stained K18 bands were cut from the gel, electroeluted, digested with trypsin followed by electrophoresis in the horizontal direction then chromatography in the vertical direction. Arrowheads indicate position where samples were spotted on the TLC plate. Mixed maps (i.e. a+b and a+c) confirmed the assignment of the peptides (not shown).

shown in Figure 1B, the relative intensity of tryptic phosphopeptide 1 increased (versus phosphopeptide 2) in G₂/M enriched HT29 cells. We have shown previously that K18 phosphopeptide 2 represents K18 Ser52 phosphorylation, which is a major physiologic K18 phosphorylation site (Ku and Omary, 1994).

We used manual Edman degradation, coupled with site-directed mutation analysis, to identify phosphoserine residue(s) in peptide 1 that were isolated from G₂/M enriched cells. Edman degradation of the purified ³²PO₄-labeled tryptic phosphopeptide 1 resulted in release of ³²P during the seventh cycle (Figure 2A). This result and the

Fig. 2. Identification of K18 Ser33 as an *in vivo* mitosis-induced phosphorylation site that can be phosphorylated *in vitro* by cdc2 kinase. **(A)** Peptide 1 from aphidicolin synchronized then mitotically-enriched cells was isolated by scraping from four cellulose TLC plates followed by extraction with water. The extracted peptide was subjected to manual Edman degradation. Released counts after each cycle are shown, as well as the counts that remained in the filter after the 10th cycle. Single letter abbreviations are used to indicate the only potential K18 peptide that has a serine at the seventh position after an arginine (based on human K18 amino acid sequence). The predicted trypsin digestion site is indicated by an arrow. **(B)** WT K18 or a Ser33→Ala K18 were co-transfected with WT K8 into NIH 3T3 cells followed by metabolic labeling with ^{32}P (5 h, 250 $\mu\text{Ci/ml}$) then isolation of K18 and tryptic peptide mapping. Equal counts of WT K18 and the Ser33→Ala K18 were spotted in map c. **(C)** K8/18 immunoprecipitates, isolated from HT29 cells, were phosphorylated *in vitro* using cdc2 kinase and γ - ^{32}P -ATP as described in Materials and methods. K18 was then isolated and subjected to tryptic peptide mapping. This panel shows a comparison of the tryptic phosphopeptides of *in vivo* labeled K18, cdc2 *in vitro* phosphorylated K18 and a mix of equal counts from each.

known amino acid sequence of human K18 (Leube *et al.*, 1986; Oshima *et al.*, 1986), coupled with previous studies, which showed that K18 phosphorylation in mitotic cells was localized to the NH_2 -terminal 125 amino acids of the head domain (Chou and Omary, 1993), indicated that Ser33 was the only likely phosphorylation site in phosphopeptide 1. This was confirmed by generating a K18

Ser33→Ala mutant and comparing the tryptic phosphopeptide map with that of wild-type (WT) K18. This comparison showed that peptide 1 phosphorylation was abolished in the K18 Ser33→Ala mutant (Figure 2B), and indicated that K18 Ser33 is a mitosis-induced phosphorylation site *in vivo*. The relatively low intensity of phosphopeptide 1 (Figure 2B, panel a) is related to the transient transfection system used and the inability to enrich for high levels of mitotic cells (not shown). Since K18 Ser33 phosphorylation increased during mitosis, we tested whether the mitosis-activated p34^{cdc2} (cdc2) kinase phosphorylated this site preferentially. As shown in Figure 2C, the peptide containing K18 Ser33 was the major phosphorylated peptide after *in vitro* phosphorylation by cdc2 kinase, which correlates with increased K18 Ser33 phosphorylation during mitosis (Figure 1B).

K18 Ser33 phosphorylation dictates binding to 14-3-3 proteins

We tested whether K18 phosphorylation plays a role in keratin binding to 14-3-3 proteins. BHK-21 cells were cotransfected with WT K8 and WT K18, or with WT K8 and K18 mutants. Transfected cells were treated with okadaic acid (OA) to induce hyperphosphorylation of K8/18 and subsequent binding of 14-3-3 proteins to K18, which in turn was probed by blotting with an anti-14-3-3 antibody. As shown in Figure 3A, treatment of transfected BHK cells with OA results in association of K18 with 14-3-3 that is comparable with that in untransfected OA-treated HT29 cells, which express endogenous K8/18 (compare lanes b and d). As expected, 14-3-3 proteins show no significant binding to K8/18 in asynchronously growing HT29 cells or in BHK cells that were transfected with WT K8/18 in the absence of OA (Figure 3A, lanes a and c). In the presence of OA, binding of 14-3-3 proteins to K8/18 is abolished if a K18 Ser33→Ala mutant or a K18 Ser33,52→Ala double mutant is used, but is not altered if a K18 Ser52→Ala mutant is transfected (Figure 3A). In addition, a glycosylation-negative (gly⁻) K18 construct (i.e. K18 Ser29,30,48→Ala; Ku and Omary, 1995) did not significantly interfere with binding of 14-3-3 proteins (Figure 3A) despite the proximity of the Ser29 and 30 glycosylation sites to the Ser33 phosphorylation site. Of note, K18 Ser33→Asp/Glu mutants do not bind 14-3-3 upon transfection (not shown, see also Figure 9B), and as such Asp/Glu do not substitute for phosphoserine. Hence, K18 Ser33 phosphorylation is essential for binding of keratin to 14-3-3 proteins, and Asp/Glu substitutions do not recapitulate binding of 14-3-3 proteins.

Characterization of phospho-Ser33 K18 in cultured cells using phosphopeptide-specific antibodies

We developed a rabbit antibody (Ab 8250) which recognized the phospho-Ser33 (pS33) K18 epitope, to study the biochemical and topographical properties of pS33 K18. Similar to the anti-pS52-K18 (Ab 3055), which was described previously (Liao *et al.*, 1995a), binding of Ab 8250 became more prominent upon G₂/M enrichment of HT29 cells (Figure 3B). This is consistent with the tryptic phosphopeptide maps that showed increased intensity of phosphopeptide 1 in G₂/M enriched cells (Figure 1B). Further confirmation of the specificity of the 8250 Ab to phospho-Ser33 K18 is based on comparable binding of

Fig. 3. Mutational analysis of K18 binding to 14-3-3 proteins, and characterization of Ab 8250. (A) WT K18 or mutant K18 were cotransfected with WT K8 into BHK cells. After 3 days, cells were incubated with OA (1 μ g/ml, 2 h), followed by immunoprecipitation of K8/18 then analysis by SDS-PAGE and staining with Coomassie Blue. Duplicate precipitates were blotted with anti-14-3-3 Ab. As a control, K8/18 immunoprecipitates from HT29 cells (with and without OA treatment) are also shown. (B) K8/18 precipitates were obtained from colcemid-treated and untreated HT29 cells (using mAb L2A1), separated by SDS-PAGE then blotted with Ab 8250 or Ab 3055, which specifically recognize a phosphopeptide of K18 Ser33 (anti-pS33) or Ser52 (anti-pS52), respectively. The small arrow indicates a hyperphosphorylated form of K8 that is generated after mitotic arrest (Liao *et al.*, 1995b). (C) NIH 3T3 cells were cotransfected with WT K8/18 or WT K8 and Ser33 or Ser52→Ala K18 mutants. K8/K18 precipitates were then prepared and blotted with Ab 8250. (D) K8/18 precipitates [identical to those used in (B)] were analyzed by isoelectric focusing (IEF) in the first dimension then SDS-PAGE in the second dimension followed by blotting with Ab 8250. The blotted membrane was stained with Coomassie Blue to match stained isoforms (I–III) with those that bound to Ab 8250 (II and III). Identical results to those obtained with Ab 8250 (B–D) were also obtained with mouse mAb IB4 which also recognizes pS33 K18 (not shown).

the antibody to WT K18 or a K18 Ser52→Ala mutant, which is abolished with a K18 Ser33→Ala mutant (Figure 3C). The anti-pS33 8250 Ab recognized the phosphorylated acidic isoforms of K18 but not the major Coomassie-stained isoform I that represents non-phosphorylated K18 (Figure 3D). Immunofluorescence staining of HT29 cells showed that Ab 8250 reactivity was abolished in the presence of the phosphopeptide that was used to generate the antibody (only a blank field was seen, not shown), but not in the presence of a nonphosphorylated form of the peptide (Figure 4a). Taken together, the immunological and molecular data indicate that Ab 8250 specifically recognizes a K18 phospho-Ser33-containing epitope.

In addition to rabbit Ab 8250, we generated a mouse mAb (IB4) which also specifically recognized pS33 K18 (see Materials and methods). IB4 and 8250 Abs had

identical reactivity patterns when tested as in Figure 3B–D (not shown). Immunofluorescence triple staining of HT29 cells, using Abs 8250 and IB4 and DNA staining, showed colocalization of 8250 and IB4 within mitotic cells and displayed a punctate staining pattern (Figure 4a–c). As shown previously (Liao *et al.*, 1995a), Ab 3055 also stained reorganized filaments in mitotic cells (Figure 4d) but there was only partial colocalization between pS33 and pS52 K18 species (Figure 4d–f).

We also compared staining of 14-3-3 proteins and keratin in HT29 cells using mAb LJ4, which recognizes mitotic cell phospho-K8 (Liao *et al.*, 1997). Abs 8250/IB4 could not be used in this setting since they bind K18 species that are unbound to 14-3-3 proteins (Figure 5B), which led us to use pS73-K8 staining as a surrogate marker for mitotic keratins. As shown in Figure 4h,

Fig. 4. Confocal fluorescence imaging of stained phospho-keratins, 14-3-3 proteins and DNA in HT29 cells. HT29 cells were triple stained for DNA (blue) and pS33 K18 using mAb IB4 and Ab 8250 (**a-c**); or for DNA, pS52 (Ab 3055), and pS33 (IB4) (**d-f**) or for DNA, pS73 K8 (LJ4) and 14-3-3 (**g-i**). Staining in **a-f** included the non-phosphorylated form of the peptide that was used for immunization (no difference with or without the peptide, not shown). Addition of the phosphopeptide used for immunization totally depletes antibody binding (not shown). All triple images were acquired from a single *z*-plane (0.45 μ m thickness). Each colored image was acquired sequentially to eliminate bleedthrough, and panels **c**, **f** and **i** show the overlay of the individually acquired green, red and blue images.

staining of 14-3-3 proteins was diffuse with a fine punctate pattern that was peripheral and throughout the cell, consistent with its enrichment in the cytosolic and NP40 fractions in these cells (Liao and Omary, 1996). Partial

colocalization of keratin and 14-3-3 proteins was noted particularly with some of the peripheral dots in mitotic cells (Figure 4g-i). Similar partial colocalization was also noted if anti-pS431 K8 was used instead of LJ4 (not shown).

Fig. 5. Association of keratin with 14-3-3 proteins in the cytosolic, NP40, Empigen and insoluble HT29 cell fractions, and correlation with S33 phosphorylation. **(A)** HT29 cells, asynchronously growing or colcemid G₂/M-enriched, were disrupted by nitrogen cavitation followed by pelleting to generate a detergent-free cytosolic fraction. The residual pellet was solubilized with 1% NP40 followed by pelleting to isolate a membrane-enriched NP40 fraction. The post-NP40 pellet was solubilized with 1% Empigen (Emp) followed by pelleting to isolate a cytoskeletal-enriched fraction. K8/18 was then precipitated from each fraction and an insoluble K8/18 fraction was prepared by adding sample buffer to the post-Empigen pellet. Precipitates were analyzed by SDS-PAGE followed by blotting with Ab 8250 (anti-pS33 blot) or with anti-14-3-3 Ab. Asterisks in (A) and (B) indicate hyperphosphorylated K8. **(B)** G₂/M-enriched HT29 cells were solubilized with 1% NP40 followed by precipitation using mAb L2A1 (lane 1) or IB4 (lane 2), which recognize the total keratin pool or the pS33 K18 pool, respectively. Alternatively, a similar NP40 lysate was used for precipitation with mAb IB4 (lane 3). Empigen was then added to the residual IB4-immunodepleted lysate to release 14-3-3 proteins that are bound to K8/18, followed by reprecipitation with mAb IB4 (lane 4) then L2A1 (lane 5). Immunoprecipitates were analyzed by SDS-PAGE, blotted with Ab 8250 and stained with Coomassie Blue.

Partitioning of phospho-Ser33 K18 species in cultured cells

The association of keratin with 14-3-3 proteins correlates with the subcellular distribution of 14-3-3 proteins, given that 14-3-3 proteins are detected only in cytosolic and NP40 fractions of mitotic and non-mitotic cells (Liao and Omary, 1996). Since Ser33 phosphorylation is essential for binding of K18 to 14-3-3 proteins (Figure 3A), we used Ab 8250 to examine the distribution of pS33 K18 in sequentially isolated cytosolic, membrane enriched (i.e. NP40-solubilized), cytoskeletal (i.e. Empigen solubilized) and remaining non-Empigen-solubilized fractions. K18 Ser33 phosphorylation is minimal in the Empigen and pellet fractions of non-mitotic cells (Figure 5A, lanes 5 and 7) as compared with the NP40 and cytosolic fractions (lanes 1 and 3). However, K18 Ser33 phosphorylation increases in all mitotic cell fractions, including the cytosolic and NP40 fractions which contain K18-bound 14-3-3 (Figure 5, lanes 2 and 4). Of note, keratins from

non-mitotic cytosolic and NP40 cell fractions exhibit low yet appreciable Ser33 K18 phosphorylation (Figure 5, lanes 1 and 3; Figure 4) based on reactivity with Ab 8250 and mAb IB4. This indicates that K18 S33 phosphorylation is necessary but not sufficient for binding of 14-3-3 proteins, and at least one other mitosis event is needed for binding of K18 to 14-3-3 proteins to occur *in vivo*.

The tetrameric nature of soluble K8/18 (i.e. two K18 and two K8 molecules) in HT29 cells (Chou *et al.*, 1993), and of other IF proteins such as vimentin (Soellner *et al.*, 1985), led us to test whether NP40-solubilized K18 that is bound to 14-3-3 proteins has a different overall Ser33 phosphorylation versus K18 pS33 that is non-14-3-3 bound. As shown in Figure 5B, mAb IB4 (lane 2) and Ab 8250 (not shown) precipitated K8/18 efficiently from mitotic cells, but these keratin species lacked associated 14-3-3 proteins in contrast with L2A1 immunoprecipitates (lane 1) that were obtained from the same cells. This indicates that binding of 14-3-3 proteins to K18 pS33

blocks IB4 and 8250 Ab binding. The stoichiometry of K18 Ser33 phosphorylation is similar when comparing K18 species that are bound or unbound to 14-3-3 proteins (Figure 5B, lanes 3 and 4). The K18 14-3-3-bound species were obtained by: immunodepleting keratins that bind IB4 [i.e. the non-(14-3-3)-bound], then releasing all 14-3-3-bound keratin in the NP40-solubilized fraction by adding Empigen (Liao and Omary, 1996) followed by re-immunoprecipitation with IB4. As anticipated, the specific activity of pS33 K18 that is isolated by precipitation with mAb IB4 is higher than that of the overall K18 pool that is isolated by precipitation with mAb L2A1 (Figure 5B, lane 5).

Partitioning of pS33 and pS52 K18 species in mouse liver

We tested whether the increased phosphorylation of K18 Ser33 that was observed in mitotic HT29 cells also occurred in the context of a tissue in regenerating mouse liver. As shown in Figure 6 (doubly stained panels a and a'), mitotic cells (e.g. cell in center of field) stain brightly with Ab 8250 in regenerating mouse liver, with similar results obtained with mAb IB4 (not shown). This was not seen in a normal liver stained with either of these two antibodies (e.g. Figure 6, panel c). Most of the cells with increased Ab 8250 staining also show new bright staining with Ab LJ4 (Figure 6, doubly stained panels b and b') which recognizes K8 pS73 in mitotic hepatocytes after partial hepatectomy (Liao *et al.*, 1997). The hepatocyte staining pattern with IB4 and 8250 Abs was filamentous-cytoplasmic, with accentuation of what appears to be apical staining. Apical enrichment of IB4 staining was verified by double staining and colocalization with aminoligopeptidase (AOP) (Figure 6, panels c and c'), an apical canalicular marker (Kartenbeck *et al.*, 1996).

Previous fluorescence staining of mouse and human liver using Ab 3055, which recognizes K18 pS52, manifested what appeared to be a basolateral staining pattern (Liao *et al.*, 1995a). However, comparison of fluorescence staining in mouse liver using mAb IB4 and Ab 3055 showed colocalization (Figure 6, panels d and d'). This indicates that the pS52 K18 species are preferentially localized within the apical domain of hepatocytes, rather than the basolateral domain, and underscores the importance of double staining using defined markers. With regard to staining of 14-3-3 proteins in hepatocytes, we did not appreciate any colocalization of keratin and 14-3-3 proteins since 14-3-3 ζ staining was mixed punctate/diffuse (Figure 6, panel e).

We examined the relative levels of pS33 K18 species in different mouse liver subcellular fractions, and the interrelationship of K18 molecules that are phosphorylated on S52 versus S33. K8/18 were isolated from mouse liver that was first solubilized with NP40 to obtain a cytosolic/membrane fraction, then solubilized with Empigen to isolate a cytoskeletal-enriched fraction (Liao *et al.*, 1996). The pS33 K18 species (isolated using Abs IB4 or 8250) represent a small but discernable portion of the NP40-solubilized keratin pool as compared with the total keratin pool that is isolated using mAb L2A1 or rabbit Ab 8592 (Figure 7A). Under these non-proliferative conditions or after partial hepatectomy, 14-3-3 proteins did not coprecipitate with K8/18 due to the overall low percentage

of dividing cells at any given time (not shown). Comparison of K18 S52 and S33 phosphorylated species that are immunoprecipitated from the NP40 or Empigen fractions, showed that K18 molecules that are phosphorylated on Ser33 are partially phosphorylated on Ser52 (Figure 7B). We were unable to carry out the reverse experiment using Ab 3055 since it does not immunoprecipitate efficiently (not shown). IEF/SDS-PAGE analysis demonstrated that mAb IB4 precipitates phosphorylated and non-phosphorylated K18, with enrichment of isoform 2 as compared with mAb L2A1 (Figure 7C, panels a and b). This indicates that during interphase, most of K8/18 tetramers contain a singly phosphorylated K18 species. The presence of primarily a single spot after blotting of two-dimensionally separated IB4 precipitates with Ab 8250 (i.e. isoform 2; Figure 7C, panel c), the relative prominence of isoform 3 when Ab 3055 is used for immunoblotting (Figure 7C, panel d) and the observation of relative limited S52 phosphorylation of pS33 K18 species (Figure 7B) further support the conclusion that a major fraction of K18 species in liver is phosphorylated on either Ser52 or Ser33 during interphase.

Effect of mutating K18 Ser33 in stably transfected cells

In order to assess the biological relevance of K18 Ser33 phosphorylation and K18 binding to 14-3-3 proteins, we first examined keratin filament staining in NIH 3T3 and BHK cells that were cotransfected with WT K8 and either WT K18, S33→A or S33→D K18. Since only rare mitotic cells were transfected, and no difference in keratin filament organization was noted in transiently transfected cells (not shown), we generated stable cell lines. Most of the clones were unstable or had low percentages of transfected cells, but one clone was stably maintained for each of the mutant constructs while several clones were obtained for the WT K18 construct. Staining of these stably transfected cells with L2A1 or 8592, which recognize the total keratin pool (not shown), or with mAb 5B3 which recognizes pS431 K8 (Ku and Omary, 1997) showed similar findings with collapse of the filaments in the majority of cells that express the Ser33 mutant as compared with normal-appearing filaments in cells that express WT K8/18 (Figure 8, panels a-c). Examination of transfected mitotic cells that express K18 Ser33→Ala/Asp, using mAb LJ4, also showed filament collapse or only a fine perinuclear keratin rim as compared with cells that express WT K18 (Figure 8, panels d-f). In this transfected cell system, no significant colocalization was noted between K8/18 and 14-3-3 proteins and no clear differences were discerned in staining of 14-3-3 proteins when comparing WT versus mutant keratin-expressing cells (Figure 8, panels d-f and d'-f'). However, this is not surprising since 14-3-3 proteins associate preferentially with the cytosolic K8/18 fraction of these transfected cells (not shown) as compared with colcemid-treated HT29 cells (Figure 5A).

Previously, we showed that recombinant 14-3-3 ζ acts *in vitro* to sequester phosphorylated, but not unphosphorylated, keratins that were purified from mitotic or interphase cells, respectively, using an *in vitro* filament assembly assay (Liao and Omary, 1996). We tested the effect of Ser33→Ala/Asp mutations in transfected cells on keratin partitioning in the cytosolic or NP40-solubilized compart-

Fig. 6. K18 Ser33 phosphorylation increases in mitotic hepatocytes and shares some topographical features with pS52 K18. (**a** and **a'**) Transgenic mice that express normal human K18 were subjected to partial hepatectomy followed by double staining of the regenerating liver with YO-PRO-1 (nuclear staining) and with Ab 8250 (pS33 K18 staining). (**b** and **b'**) Mouse regenerating liver was double stained with mAb LJ4, which recognizes mitosis-induced K8 pS73, and with Ab 8250. (**c** and **c'**) Normal mouse liver was double stained with rabbit anti-amino-oligopeptidase (AOP) and with mAb IB4 (anti-pS33 K18). Note colocalization of the apically enriched IB4⁺ staining with AOP. (**d** and **d'**) Mouse regenerating liver was double stained with mAb IB4 and Ab 3055 (anti-pS52 K18). Note the increased staining in the mitotic hepatocyte. (**e**) Mouse regenerating liver was stained with anti-14-3-3ζ Ab.

ments using two approaches. First, the cytosolic and NP40 fractions were compared between OA-treated and untreated cells in WT, S33A and S33D K18 expressing cells. We used OA since the stably transfected cells could not be mitotically enriched to a degree that allowed meaningful biochemical analysis. The overall average keratin expression as determined by immunoblotting of total cell lysates was nearly 2-fold lower for the S33A clone as compared with the other two clones (Figure 9A) and the percent of cells that expressed K8/18 was ~40% in all three clones (not shown). Addition of OA increased

cytosolic keratin in the three clones as would be expected by keratin hyperphosphorylation (Figure 9A). An OA-induced increase in keratin partitioning with the NP40 fraction was also noted in WT K18-expressing cells, but not in the two K18 mutant clones (Figure 9A). These distinct differences correlate with the altered keratin staining profile in the K18 mutant cells as compared with WT K18-expressing cells (Figure 8). In the second approach, we transiently cotransfected 14-3-3ζ and ε into the three stable clones to assess the effect of overexpression of 14-3-3 proteins on K18 partitioning into the NP40 fraction.

Fig. 7. Analysis of K18 Ser33 and Ser52 phosphorylation in mouse liver. (A) Liver tissue was removed from transgenic mice that express WT human K18, solubilized with 1% NP40 followed by pelleting of the non-solubilized fraction. The pellet was then solubilized with 1% Empigen (Emp) followed by centrifugation. Equal amounts were then taken from the NP40 and Emp fraction and used for immunoprecipitation followed by analysis of equal fractions of the precipitates by SDS-PAGE then staining with Coomassie Blue. The antibodies used for precipitation were: mAb L2A1 or rabbit Ab 8592 (which recognize the total keratin pool), and mAb IB4 or rabbit Ab 8250 (anti-pS33 K18). (B) Mouse liver NP40 or Emp fractions were isolated as in (A) followed by precipitation using mAb L2A1 (lanes 1 and 4) or IB4 (lanes 2 and 5). The remaining lysates (after precipitation with IB4) were used for reprecipitation with mAb L2A1 (lanes 3 and 6). Nearly equal amounts of each immunoprecipitate (i.p.) were blotted using 8250 or 3055 Abs. (C) K8/18 precipitates were obtained using mAb L2A1 or IB4 from the NP40-solubilized mouse liver fraction. Precipitates were analyzed by IEF in the horizontal dimension then SDS-PAGE in the vertical dimension, followed by staining with Coomassie Blue. Duplicate samples of IB4 precipitates were also analyzed by blotting with Ab 8250 or 3055. Assignment of isoforms 1-3 was done by two-dimensional analysis of mixed and equal amounts of the L2A1 and IB4 i.p.s (not shown). Note that only the phosphorylated acidic K18 isoforms 2 and 3 are recognized by Abs 8250 and 3055. Phosphorylation of only the relatively acidic K8/18 isoforms has been established previously (Liao *et al.*, 1996).

Overexpression of 14-3-3 proteins varied among the three clones but even a small increase in the levels of 14-3-3 proteins in WT-expressing cells was associated with a small but reproducible (~2-fold) increase in K8/18 that was

immunoprecipitated from 14-3-3 proteins versus control vector transfected cells (Figure 9B). The pS33 binding Abs (i.e. 8250 and IB4) do not recognize Ser33→Asp K18 (Figure 9A), which in turn does not bind to 14-3-3

Fig. 8. Immunofluorescence staining of NIH 3T3 cells stably transfected with WT or Ser33-mutant K18. NIH 3T3 cells were stably cotransfected with WT K8 and one of three K18 constructs: WT K18, K18 Ser33→Ala (S33A) or K18 Ser33→Asp (S33D). Cells were plated onto cover slips and after 24 h were stained with antibodies to K8 pS431 (mAb 5B3) (a–c) or double stained with mAb LJ4 (anti-pS73 K8) and anti-14-3-3 proteins (d and d'; e and e'; f and f'). Identical cells in the double stained panels are highlighted by arrows. The remaining four clones that expressed WT K18 had filament assembly similar to that in (a) and (d) (not shown).

proteins (Figure 9B) (despite long exposure of the blots after gel loading a mutant:WT immunoprecipitate ratio of 2:1, not shown). Taken together, these data indicate that K18 Ser33 plays an important role in binding of 14-3-3 proteins and in K18 subcellular organization and distribution.

Discussion

Characterization of K18 Ser33 phosphorylation as a regulatory modification for binding of 14-3-3 proteins

We used biochemical and mutational analysis to demonstrate that K18 Ser33 is phosphorylated *in vivo*. Identification of the K18 phosphorylated sequence ²⁶RPV-SSAASVY enabled generation of antibodies that specifically recognized pS33 K18 (Figures 3 and 5). Although proximal K18 S29 and S30 are known glycosylation sites (Ku and Omary, 1995), inhibition of glycosylation at these sites by Ser→Ala mutations did not significantly affect binding of keratin of 14-3-3 proteins (Figure 3A). The kinase(s) involved in K18 Ser33 phosphorylation remain to be identified. Preferential *in vitro* phosphorylation of K18 Ser33 by cdc2 kinase, as compared with other sites

(Figure 2C), makes it an attractive candidate kinase during mitosis. However, K18 Ser33 phosphorylation during interphase suggests alternate or additional kinases, unless the turnover of this site during interphase is low.

Binding of 14-3-3 proteins to K18 requires Ser33 and its phosphorylation, and in this context negatively charged amino acids such as aspartate and glutamate do not substitute for K18 Ser33 phosphorylation. Several lines of evidence support our conclusion that the K18 pS33-containing motif is involved, in a phosphorylation-dependent fashion, in binding to 14-3-3 proteins: (i) K8/18 binding to 14-3-3 proteins occurs only under conditions that involve keratin hyperphosphorylation including mitosis, subjecting cells to heat or OA treatment (Liao and Omary, 1996), or under transfection conditions with OA-induced hyperphosphorylation (Figures 3A, 5 and 9B); (ii) binding of 14-3-3 proteins to the K8/18 complex occurs with K18, and not with K19 or K8, as determined by *in vitro* reconstitution of the binding. In addition, phosphatase pretreatment of K8/18 immunoprecipitates, isolated from mitotic cells but not from interphase cells, prevents binding of 14-3-3 proteins (Liao and Omary, 1996); (iii) although both major K18 phosphorylation sites (i.e. S33 and S52) undergo increased phosphorylation

Fig. 9. Effect of K18 Ser33→Ala/Asp mutations in transfected cells. (A) Stably transfected cells, used in Figure 8, were cultured in the presence or absence of OA for 2 h. Equivalent fractions (1/10) of the cells were solubilized with sample buffer (total cell lysate) and the remaining portion was freeze-thawed three times followed by centrifugation to generate a cytosolic fraction. The pellet was solubilized with 1% NP40 followed by pelleting. Equal fractions (15 µg) were then analyzed by blotting using Ab 8592 which recognizes the total keratin pool (K8/18 blot) or Ab 8250 (pS33 blot). (B) Stably transfected cells used in (A) were cotransfected with 14-3-3 ϵ and ζ , or with vector alone. After 3 days, transfected cells were cultured in the presence of OA (1 µg/ml) for 2 h, after which total lysates were prepared from a fraction of the cells, as in (A), and the remaining cells were solubilized with 1% NP40 followed by precipitation of K8/18 using mAb L2A1. Equivalent fractions of the precipitates were separated by SDS-PAGE followed by staining with Coomassie Blue, or were blotted with anti-14-3-3 Ab. Similarly, equal fractions of the total lysates were blotted using anti-14-3-3 Ab. The FLAG-tagged 14-3-3 ζ (indicated by an asterisk) migrates slightly slower than the endogenous isoform. Similar results to those shown in (A) and (B) were obtained in two other separate experiments.

during mitosis (Figures 1B and 3B), it is only the K18 Ser33→Ala, and not the Ser52→Ala, mutation that totally prevents binding of 14-3-3 proteins (Figure 3A) and (iv) two antibodies that recognize K18 pS33 (Abs 8250 and IB4) do not co-immunoprecipitate 14-3-3 proteins (Figure 5B), which indicates that K18-bound 14-3-3 proteins cannot be recognized by these two antibodies but are recognized by several other mAbs that bind K8 or K18 (Figure 1A and data not shown).

Binding of 14-3-3 proteins to phospho-serine motifs

The only reported sequences demonstrated to be 14-3-3-binding phospho-motifs are listed in Table I. In addition,

double mutation of the underlined serines in the sequence ...RHSLPFSLP... or ...RLGSTFSLD... of the Cbl protein abrogated binding of 14-3-3 proteins *in vitro*, although the underlined serines were not individually mutated and their phosphorylation was not examined (Liu *et al.*, 1997). The commonality between the BAD, Raf-1, and cdc25 motifs is an RXXSXP, while one of the two phosphorylation sites that regulate binding of 14-3-3 proteins in PTPH1 lack the proline (see Table I). Interestingly, the K18 S33-containing motif appears very different to the 14-3-3 binding phospho-motifs that are presently known. One potentially common feature between K18 pS33 and the Cbl-serines that are important for binding of 14-3-3 proteins is RX₁₋₂SX₂₋₃S (Liu *et al.*, 1997). If this motif is indeed found to be important for binding of 14-3-3 proteins to other proteins, then phosphorylation of only the last serine in this motif becomes essential for binding. Other common features among all identified 14-3-3-binding protein sequences to date is a serine-rich environment and relative proximity to an arginine/proline, which would provide a favorable kinase phosphorylation site. In addition to K18 Ser33 phosphorylation, it appears that another event is also important for binding of K18 to 14-3-3 proteins. This is based on finding basal K18 Ser33 phosphorylation during interphase, with abundant cytoplasmic and membrane-associated 14-3-3 proteins, yet without any significant detectable binding of 14-3-3 proteins.

The nature of the additional event(s) that regulates binding of K18 to 14-3-3 proteins remains open to investigation, but several possibilities can be considered that involve 'unmasking' of basally phosphorylated K18 Ser33, or modifications of K18, 14-3-3 proteins, K8 and/or other participants. First, although an additional K18 modification is possible, other K18 phosphorylation sites are less likely to be involved since the phospho-peptide maps of K18 obtained under K18/14-3-3 binding conditions do not support major dephosphorylation or phosphorylation of other peptides (aside from the Ser33/52-containing peptides), and K18 glycosylation (Chou *et al.*, 1992; Ku and Omary, 1995) does not appear to be a factor (Figure 3). Secondly, regulation of binding of 14-3-3 proteins to associated proteins is possible by modification of the 14-3-3 proteins. To date, known *in vivo* phosphorylation sites of 14-3-3 proteins are Ser185 (Aitken *et al.*, 1995) and Thr/Ser233 (Dubois *et al.*, 1997), and it appears that dephosphorylation of 14-3-3 Thr233 may promote binding to Raf. Similarly, it appears that keratin-bound 14-3-3 proteins are unphosphorylated (based on metabolic labeling, not shown). Hence, dephosphorylation of 14-3-3 proteins may be a potential mechanism that helps regulate binding of 14-3-3 proteins to their associated proteins. Alternatively, even though it is estimated that only ~6% of cellular 14-3-3 ζ is bound to K8/18 during mitosis (Liao and Omary, 1996), it is possible that during interphase most if not all of the 14-3-3 proteins are occupied by their other multiple binding partners. Thirdly, K8 may also play a role in binding of 14-3-3 proteins to K8/18 particularly since its phosphorylation increases during mitosis (Celis *et al.*, 1983; Chou and Omary, 1994). For example, K8 increases its phosphorylation or becomes newly phosphorylated during mitosis on Ser431 (Ku and Omary, 1997) and Ser73 (Liao *et al.*, 1997), respectively. However,

Table I. Phosphomotifs of 14-3-3 binding proteins

Protein	Sequence	Motif	Reference
Raf-1	²⁵⁶ RSTSTP	R S X S X P	Michaud <i>et al.</i> , 1995; Muslin <i>et al.</i> , 1996
Raf-1	⁶¹⁸ RSASEP	R S X S X P	Muslin <i>et al.</i> , 1996
BAD	¹⁰⁷ RSRHSSYP	R X R X X S X P	Zha <i>et al.</i> , 1996
BAD	¹³¹ RGRSRAP	R X R S X S X P	Zha <i>et al.</i> , 1996
Cdc25	²¹³ RSPSMP	R S X S X P	Peng <i>et al.</i> , 1997
PTPH1	³⁵⁶ RSLSVE and ⁸³² RVDSEP	R X X S	Zhang <i>et al.</i> , 1997
Keratin 18	²⁶ RPVSSAASVY	Unique (ser-rich)	this manuscript

Single letter abbreviations are used to denote the amino acid sequence of the phosphorylated domains and motifs that are based on these sequences. Phosphorylated serines are indicated by italic bold 'S', and the letter 'X' corresponds to any amino acid. PTP, protein tyrosine phosphatase.

mutation of these two sites does not inhibit binding of 14-3-3 proteins in transfected OA-treated cells (not shown). Fourthly, unmasking pS33 K18 by release of an associated protein such as heat-shock protein (hsp) 70 (Liao *et al.*, 1995c) or another putative protein is a theoretical consideration. This possibility is unlikely to play an important role since hsp70 is the only other stoichiometrically significant associated protein, which binds to K8 (Liao *et al.*, 1995c) independent of binding of 14-3-3 proteins (Liao *et al.*, 1996). Finally, the K18 specific activity of phosphorylated Ser33 is similar whether it is bound to 14-3-3 proteins or unbound (Figure 5B), which excludes the possibility that binding of 14-3-3 proteins to K18 occurs preferentially with K8/18 tetramers that have both K18 molecules phosphorylated on Ser33.

Segregation of pS52 and pS33 K18 species

The use of phosphopeptide specific antibodies as probes of IF protein phosphorylation has afforded a powerful tool for studying *in vivo* protein phosphorylation (reviewed by Inagaki *et al.*, 1997). Ab 3055 (anti-pS52 K18, Liao *et al.*, 1995a) and the generation of rabbit and mouse Abs to pS33 K18 (this work) facilitated studying these two phosphorylation sites and their interrelationship in tissues. Staining using these anti-phospho-K18 antibodies showed partial colocalization of the phosphorylated K18 species at the hepatocyte apical membrane (Figure 6) and in cultured HT29 (particularly mitotic) cells (Figure 4). At the biochemical level, significant segregation of K18 molecules that are phosphorylated on Ser52, as compared with Ser33, was noted in mouse liver tissue (Figure 7). Apical enrichment of pS52 K18 in the liver is different to what we previously assigned as basolateral staining, since double staining with well-recognized markers had not been performed. However, the apical enrichment of pS52 K18 in liver is compatible with what was seen in the pancreas (Liao *et al.*, 1995a), which is also noted by double staining of IB4 (anti-pS33) and 3055 (anti-pS52) antibodies in the pancreas (not shown).

The presence of individual K8/18 tetramers that are phosphorylated on either K18 Ser33 or Ser52 supports the hypothesis that each modification is likely to have separate functions (Omary *et al.*, 1998). For Ser52 phosphorylation, transfection experiments indicated that this phosphorylation site is important for filament reorganization during mitotic arrest or upon treatment with okadaic acid (Ku and Omary, 1994). In the case of K18 Ser33 phosphorylation, one important role is allowing binding of 14-3-3 proteins to K8/18 during mitosis. However,

basal K18 Ser33 phosphorylation in tissues and cultured cells (Figures 4–7) suggests that Ser33 phosphorylation may have additional role(s) during interphase. The basally phosphorylated K18 S33 and S52 species are localized preferentially in the soluble cytosolic and NP40-solubilized compartments (Figures 5 and 7) as compared with the Empigen and post-Empigen pellet fractions. Hence, the phosphorylation gradient that was observed in cultured cell lines (Liao *et al.*, 1996) also occurs in tissues (Figure 7). Furthermore, the filamentous decoration of K8/18 by anti-pS33 antibodies (Figures 4 and 6), coupled with the ease of extractability of most of pS33 K18 by NP40, indicates that this phosphopeptide probably decorates the outside of the filaments and hence is likely to be involved in interacting with cytoplasmic and/or membrane components rather than be involved in filament packing. Of note, the keratin phosphorylation gradient between cell cytosol and cytoskeleton is not uniform for all phosphorylation sites. For example, pS73 K8 is nearly evenly distributed between the cytosolic and pellet fractions in G₂/M-arrested cells (Figure 5A).

Significance of binding of K18 to 14-3-3 proteins

Mutational analysis of K18 in transient transfection systems (NIH 3T3 and BHK cells, not shown) did not reveal a functional role for K18 S33 phosphorylation and subsequent binding of 14-3-3 proteins. However, stable transfection of K18 Ser33→Ala/Asp resulted in keratin filament collapse which was not seen in WT K8/18 stably transfected cells (Figure 8). This indirectly suggests that the filament collapse is most likely to occur in cells that have undergone one or more rounds of cell division, and that K18 S33 phosphorylation (with or without association of 14-3-3 proteins) plays a role in filament organization in such cells. The mechanism that underlies this collapse remains to be investigated.

The ratio of partitioning of K8/18-associated 14-3-3 proteins between the cytosol and NP40-soluble compartments appears to vary depending on the cell system. For example, 14-3-3 proteins associate with K18 preferentially in the cytosolic compartment of aphidicolin-synchronized G₂/M cells (Liao and Omary, 1996), and OA treated HT29 and WT K8/18-transfected NIH 3T3 cells (not shown). In contrast, keratin-bound 14-3-3 proteins are preferentially found in the NP40-containing keratin compartment in colcemid-arrested cells (Figure 5A). These differences probably reflect the nature of the cells and the manipulations that were used. Regardless, our data also indicate that K18 Ser33 (and most probably its phosphorylation,

based on the results obtained upon cell treatment with OA) also plays an important role in mobilizing keratins to the NP40-solubilized compartment (Figure 9A). This role appears to be specific to the NP40-solubilized compartment, since OA treatment of K18 Ser33→Ala/Asp cell transfectants caused a significant shift of keratins to the cytosol, as was noted for the WT K18 transfected cells (Figure 9A). The increase in cytosolic K8/18 in WT and mutant K18 OA-treated cell transfectants is probably related to increased keratin phosphorylation at multiple K18 and K8 sites (not shown). The role of 14-3-3 proteins in mobilizing WT K18 but not K18 Ser33→Ala/Asp to an NP40 compartment upon OA treatment was supported by transfection of 14-3-3 proteins into WT or Ser33→Ala/Asp K18-expressing cells, with a resultant increase in the NP40-associated keratin fraction in WT but not mutant K18 expressing cells (Figure 9B). The functional significance of such mobilization remains to be examined but several attractive possibilities can be envisioned, such as allowing molecular complexes to be in close proximity at specific subcellular locations or playing a role in mitosis-associated subcellular reorganization.

Materials and methods

Reagents

$^{32}\text{PO}_4$ and $[\gamma\text{-}^{32}\text{P}]$ ATP (3000 Ci/mmol) were purchased from DuPont-New England Nuclear (Wilmington, DE). Other reagents used were: aphidicolin and colcemid (Sigma Chemical Co., St Louis, MO), cdc2 kinase (New England Biolabs, Cambridge, MA), trypsin (Worthington Biochemical Corp.), okadaic acid (LC Laboratories, Woburn, MA), YO-PRO-1 iodide and Toto-3 iodide (Molecular Probes, Eugene, OR), TransformerTM mutagenesis kit (Clontech, Palo Alto, CA) and Lipofect-AMINE liposomes (Gibco BRL, Gaithersburg, MD). Antibodies used were: mouse mAb L2A1 against human K18 (Chou *et al.*, 1992); mouse mAbs LJ4 and 5B3 against K8 pS73 (Liao *et al.*, 1997) and pS431 (Ku and Omary, 1997), respectively; rabbit Ab 3055, which recognizes K18 pS52 (Liao *et al.*, 1995a) and rabbit Abs to all 14-3-3 isoforms and to 14-3-3 ζ (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Rabbit Ab to amino-oligopeptidase (AOP) was kindly provided by Gary Gray (Stanford University). FLAG-epitope tagged 14-3-3 ζ and 14-3-3 ϵ cDNAs were kindly provided by Haiyan Fu (Emory University) and David Beach (Cold Spring Harbor Laboratory), respectively.

Cell culture and transfection

HT29 (human colon), NIH 3T3 (mouse fibroblast) and BHK-21 (hamster kidney) were from the American Type Culture Collection (Rockville, MD) and were cultured as recommended by the supplier. To obtain G₂/M cell cycle stage enriched HT29 cells, cells (~50% confluent) were treated with colcemid (0.5 $\mu\text{g}/\text{ml}$) for 36 h. Alternatively, cells were treated with aphidicolin (5 $\mu\text{g}/\text{ml}$) for 24 h followed by washing and incubation with fresh medium for 10 h. Cell cycle analysis was done by fixing cells in 100% methanol then staining with propidium iodide as described (Chou and Omary, 1993). For transient expression in NIH 3T3 or BHK-21 cells, WT K18 or K18 mutants generated with a TransformerTM mutagenesis kit were subcloned downstream of the hCMV promoter in the pMRB101 mammalian expression vector and were transiently or stably transfected into NIH 3T3 or BHK cells by using Lipofect-AMINE as described (Ku and Omary, 1994).

Biochemical and radiolabeling methods

For tryptic phosphopeptide mapping, K8/18 immunoprecipitates were obtained, using mAb L2A1, from cells labeled metabolically with $^{32}\text{PO}_4$. HT29 or transfected NIH 3T3 cells were labeled with $^{32}\text{PO}_4$ (250 $\mu\text{Ci}/\text{ml}$) for 5 h in phosphate-free RPMI 1640 medium (100 μM total PO_4). *In vitro* kinase assays were done using K8/18 immunoprecipitates that were boiled for 90 s to abolish endogenous kinase activity. Phosphorylation was performed using 5 U cdc2 kinase in 50 μl of 50 mM Tris-HCl (pH 7.4) containing $[\gamma\text{-}^{32}\text{P}]$ -ATP (10 μCi) and ATP (40 μM) for 15 min at 30°C. K18 was separated from K8 using preparative SDS-PAGE, followed by trypsin digestion and two-dimensional peptide

mapping (Boyle *et al.*, 1991). Manual Edman degradation was performed for 10 cycles on $^{32}\text{PO}_4$ -labeled peptides that were scraped off the cellulose TLC plate then extracted with water as described (Ku and Omary, 1995). For Western blotting to detect 14-3-3 proteins, HT29 cells, or NIH 3T3 and BHK cells transfected with WT K8/K18, WT K8/mutant K18 or 14-3-3 proteins were incubated in the presence or absence of OA (1 $\mu\text{g}/\text{ml}$) for 2 h. K8/18 immunoprecipitates were then obtained and analyzed by SDS-PAGE followed by blotting with anti-14-3-3 Ab. IEF was performed using a 1:4 mix of ampholines pH 3–10 and pH 5–8 (Liao *et al.*, 1996). The gels were run at 750 V for 3.5 h.

Immunofluorescence, cell and tissue staining

HT29 and transfected NIH 3T3 cells (~50% confluent) were grown on cover-slips, fixed with methanol (–20°C, 3 min), and then incubated with mAb L2A1, LJ4, 5B3 or IB4 (1:40–1:100 ascites dilution), 3055 (10 $\mu\text{g}/\text{ml}$), Ab 8250 (1:1000) or anti-AOP (1:50). Ab 8250 or IB4 were preincubated with the non-phosphorylated or phosphorylated peptides that were used for immunization (200 $\mu\text{g}/\text{ml}$, 30 min, 22°C). Cells were washed and double-stained with the DNA-staining dye YO-PRO-1 (1:20 000) and Texas red conjugated goat anti-mouse or anti-rabbit antibodies (1:100). Double staining with two primary antibodies was carried out using antibodies from two species and the second stage antibodies were preabsorbed to ensure specificity to the species of the primary antibody. Triple staining was done using mouse and rabbit primary antibodies and Toto-3 DNA staining dye (1:20 000). Fluorescence was analyzed using Bio-Rad or Leica confocal microscopes. Partial hepatectomy and immunofluorescence staining of mouse liver was done as described (Liao *et al.*, 1997). Post-hepatectomized livers were obtained 60 h after a 66% hepatectomy.

Anti-phosphopeptide antibody generation

The peptide RPVSSAapSVYAGAC was synthesized commercially (AnaSpec Inc., San Jose, CA) then coupled to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. This peptide corresponds to human K18 amino acids 26–38, with a terminal cysteine added for coupling. The sequence is identical to mouse K18 except that human K18 val28 is replaced by alanine in the mouse sequence (Oshima *et al.*, 1986). Mice and rabbits were then immunized monthly, initially using complete Freund's adjuvant then incomplete adjuvant, with resultant high titer antibody after four immunizations. Mice were boosted intraperitoneally followed by fusion of the mouse spleen and myeloma cells (Kohler and Milstein, 1975). One hybridoma clone (IB4) reacted preferentially with keratins isolated from OA-treated cells, as compared with basally phosphorylated keratins, and was chosen for further characterization. The isotype of IB4 is IgG1.

Immunoprecipitation

Cells (unlabeled or metabolically prelabeled with $^{32}\text{PO}_4$) were solubilized (30 min, 4°C) with 1% Nonidet P40 in PBS supplemented with an inhibitor cocktail (Liao and Omary, 1996). After pelleting, the supernatant was used for immunoprecipitation with L2A1, IB4, 8592 or 8250 Abs that were conjugated to protein A–Sepharose. Alternatively, cells were disrupted by nitrogen cavitation or three cycles of freeze–thaw followed by pelleting to isolate a cytosolic fraction. The post-cytosolic pellet was solubilized with 1% NP40 in PBS (30 min, 4°C) followed by centrifugation to isolate a membrane fraction. In some experiments, the post-NP40 pellet was then solubilized with 1% Empigen in PBS plus protease inhibitors (Lowthert *et al.*, 1995) to obtain a cytoskeletal fraction. The cytosolic, NP40 and Empigen fractions were then used for precipitation, while the post-Empigen pellet was analyzed directly, using SDS-PAGE, by solubilizing in sample buffer (Laemmli, 1970). Solubilization of mouse liver, immuno-precipitation and staining were done as described (Ku *et al.*, 1995).

Acknowledgements

We are very grateful to Drs David Beach and Haiyan Fu for providing the 14-3-3 ϵ and ζ cDNAs, respectively; Dr Gary Gray for providing the AOP antibody; Kris Morrow for preparing the figures; Ruthie Stevens for preparing the manuscript; Evelyn Resurreccion for cutting and staining of the tissue sections; Jim Hayden and Frank Lie for assistance with confocal microscopy; Lori Lowthert and Dan Price for assistance with antibody generation and screening; and Phuoc Vo for performing the fusion. This work was supported by Veterans Administration Career Development Award, NIH Grants DK47918 and DK52951, and Digestive Disease Center Grant DK38707.

References

- Aitken, A. (1995) 14-3-3 proteins on the MAP. *Trends Biochem. Sci.*, **20**, 95–97.
- Aitken, A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathway. *Trends Cell Biol.*, **6**, 341–347.
- Aitken, A., Howell, S., Jones, D., Madrazos, J. and Patel, Y. (1995) 14-3-3 α and δ are the phosphorylated forms of Raf-activating 14-3-3 β and ζ . *J. Biol. Chem.*, **270**, 5706–5709.
- Banik, U., Wang, G.-A., Wagner, P.D. and Kaufman, S. (1997) Interaction of phosphorylated tryptophan hydroxylase with 14-3-3 proteins. *J. Biol. Chem.*, **272**, 26219–26225.
- Bonnefoy-Berard, N., Liu, Y.-C., von Willebrand, M., Sung, A., Elly, C., Mustelin, T., Yoshida, H., Ishizaka, K. and Altman, A. (1995) Inhibition of phosphatidylinositol 3-kinase activity by association with 14-3-3 proteins in T cells. *Proc. Natl Acad. Sci. USA*, **92**, 10142–10146.
- Boyle, W.J., Van Der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.
- Brasemann, S. and McCormick, F. (1995) BCR and RAF form a complex *in vivo* via 14-3-3 proteins. *EMBO J.*, **14**, 4839–4848.
- Celis, J.E., Larsen, P.M., Fey, S.J. and Celis, A. (1983) Phosphorylation of keratin and vimentin polypeptides in normal and transformed mitotic human epithelial amnion cells: behavior of keratin and vimentin filaments during mitosis. *J. Cell Biol.*, **97**, 1429–1434.
- Chou, C.-F. and Omary, M.B. (1993) Mitotic-arrest associated enhancement of O-linked glycosylation and phosphorylation of human keratins 8 and 18. *J. Biol. Chem.*, **268**, 4465–4472.
- Chou, C.-F. and Omary, M.B. (1994) Mitotic arrest with anti-microtubule agents or okadaic acid is associated with increased glycoprotein terminal GlcNAc's. *J. Cell Sci.*, **107**, 1833–1843.
- Chou, C.-F., Smith, A.J. and Omary, M.B. (1992) Characterization and dynamics of O-linked glycosylation of human cytokeratin 8 and 18. *J. Biol. Chem.*, **267**, 3901–3906.
- Chou, C.-F., Riopel, C.L., Rott, L.S. and Omary, M.B. (1993) A significant soluble keratin fraction in 'simple' epithelial cells: Lack of an apparent phosphorylation and glycosylation role in keratin solubility. *J. Cell Sci.*, **105**, 433–445.
- Conklin, D.S., Galaktionov, K. and Beach, D. (1995) 14-3-3 proteins associate with cdc25 phosphatases. *Proc. Natl Acad. Sci. USA*, **92**, 7892–7896.
- Dubois, T., Rommel, C., Howell, S., Steinhussen, U., Soneji, Y., Morrice, N., Moelling, K. and Aitken, A. (1997) 14-3-3 is phosphorylated by casein kinase I on residue 233. *J. Biol. Chem.*, **272**, 28882–28888.
- Freed, E., Symons, M., Macdonald, S.G., McCormick, F. and Ruggieri, R. (1994) binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science*, **265**, 1713–1716.
- Fu, H., Xia, K., Pallas, D.C., Cui, C., Conroy, K., Narsimhan, R.P., Mamont, H., Collier, R.J. and Roberts, T.M. (1994) Interaction of the protein kinase Raf-1 with 14-3-3 proteins. *Science*, **266**, 126–129.
- Fuchs, E. (1996) The cytoskeleton and disease: genetic disorders of intermediate filaments. *Annu. Rev. Genet.*, **30**, 197–231.
- Fuchs, E. and Weber, K. (1994) Intermediate filaments: structure, dynamics, function and disease. *Annu. Rev. Biochem.*, **63**, 345–382.
- Furukawa, Y., Ikuta, N., Omata, S., Yamauchi, T., Isobe, T. and Ichimura, T. (1993) Demonstration of the phosphorylation-dependent interaction of tryptophan hydroxylase with the 14-3-3 protein. *Biochem. Biophys. Res. Commun.*, **194**, 144–149.
- Ichimura, T., Isobe, T., Okuyama, T., Takahashi, N., Araki, K., Kuwano, R. and Takahashi, Y. (1988) Molecular cloning of cDNA for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *Proc. Natl Acad. Sci. USA*, **85**, 7084–7088.
- Inagaki, M., Inagaki, N., Takahashi, T. and Takai, Y. (1997) Phosphorylation-dependent control of structures of intermediate filaments: a novel approach using site- and phosphorylation state-specific antibodies. *J. Biochem.*, **121**, 407–414.
- Irvine, A.D. et al. (1997) Mutations in cornea-specific keratin K3 or K12 genes cause Meesmann's corneal dystrophy. *Nature Genet.*, **16**, 184–187.
- Kartenbeck, J., Leuschner, U., Mayer, R. and Keppler, D. (1996) Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. *Hepatology*, **23**, 1061–1066.
- Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**, 495–497.
- Ku, N.-O. and Omary, M.B. (1994) Identification of the major physiologic phosphorylation site of human keratin 18: potential kinases and a role in filament reorganization. *J. Cell Biol.*, **127**, 161–171.
- Ku, N.-O. and Omary, M.B. (1995) Identification and mutational analysis of the glycosylation sites of human keratin 18. *J. Biol. Chem.*, **270**, 11820–11827.
- Ku, N.-O. and Omary, M.B. (1997) Phosphorylation of human keratin 8 *in vivo* at conserved head domain serine 23 and at epidermal growth factor-stimulated tail domain serine 431. *J. Biol. Chem.*, **272**, 7556–7564.
- Ku, N.-O., Michie, S., Oshima, R.G. and Omary, M.B. (1995) Chronic hepatitis, hepatocyte fragility, and increased soluble phosphoglycokeratins in transgenic mice expressing a keratin 18 conserved arginine mutant. *J. Cell Biol.*, **131**, 1303–1314.
- Ku, N.-O., Liao, J., Chou, C.-F. and Omary, M.B. (1996) Implications of intermediate filament protein phosphorylation. *Cancer Metastasis Rev.*, **15**, 429–444.
- Ku, N.-O., Wright, T.L., Terrault, N.A., Gish, R. and Omary, M.B. (1997) Mutation of human keratin 18 in association with cryptogenic cirrhosis. *J. Clin. Invest.*, **99**, 19–23.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Leube, R.E., Bosch, F.X., Romano, V., Zimblemann, R., Höfler, H. and Franke, W.W. (1986) Cytokeratin expression in simple epithelia. III. Detection of mRNAs encoding human cytokeratins nos 8 and 18 in normal and tumor cells by hybridization with cDNA sequences *in vitro* and *in situ*. *Differentiation*, **33**, 69–85.
- Liao, J. and Omary, M.B. (1996) 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. *J. Cell Biol.*, **133**, 345–358.
- Liao, J., Lowthert, L.A., Ku, N.-O., Fernandez, R. and Omary, M.B. (1995a) Dynamics of human keratin 18 phosphorylation: Polarized distribution of phosphorylated keratins in simple epithelial tissues. *J. Cell Biol.*, **131**, 1291–1301.
- Liao, J., Lowthert, L.A. and Omary, M.B. (1995b) Heat stress or rotavirus infection of human epithelial cells generates a distinct hyperphosphorylated form of keratin 8. *Exp. Cell Res.*, **219**, 348–357.
- Liao, J., Lowthert, L.A., Ghori, N. and Omary, M.B. (1995c) The 70-kDa heat shock proteins associate with glandular intermediate filaments in an ATP-dependent manner. *J. Biol. Chem.*, **270**, 915–922.
- Liao, J., Ku, N.-O. and Omary, M.B. (1996) Two-dimensional gel analysis of glandular keratin intermediate filament phosphorylation. *Electrophoresis*, **17**, 1671–1676.
- Liao, J., Ku, N.-O. and Omary, M.B. (1997) Stress, apoptosis, and mitosis induce phosphorylation of human keratin 8 at ser73 in tissues and cultured cells. *J. Biol. Chem.*, **272**, 17565–17573.
- Liu, Y.-C., Elly, C., Yoshida, H., Bonnefoy-Berard, N. and Altman, A. (1996) Activation-modulated association of 14-3-3 proteins with Cbl in T cells. *J. Biol. Chem.*, **271**, 14591–14595.
- Liu, Y.-C., Liu, Y., Elly, C., Yoshida, H., Lipkowitz, S. and Altman, A. (1997) Serine phosphorylation of Cbl induced by phorbol ester enhances its association with 14-3-3 proteins in T cells via a novel serine-rich 14-3-3-binding motif. *J. Biol. Chem.*, **272**, 9979–9985.
- Lowthert, L.A., Ku, N.-O., Liao, J., Coulombe, P.A. and Omary, M.B. (1995) Empigen BB: a useful detergent for solubilization and biochemical analysis of keratins. *Biochem. Biophys. Res. Commun.*, **206**, 370–379.
- McLean, W.H.I. and Lane, E.B. (1995) Intermediate filaments in disease. *Curr. Opin. Cell Biol.*, **7**, 118–125.
- Michaud, N.R., Fabian, J.R., Mathes, K.D. and Morrison, D.K. (1995) 14-3-3 is not essential for Raf-1 function: Identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. *Mol. Cell. Biol.*, **15**, 3390–3397.
- Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell*, **31**, 11–24.
- Moore, B.W. and Perez, V.J. (1967) Specific acidic proteins of the nervous system. In Carlson, F.D. (ed.), *Physiological and Biochemical Aspects of Nervous Integration*. Prentice-Hall, Englewood Cliffs, NJ, pp. 343–359.
- Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.
- Omary, M.B. and Ku, N.-O. (1997) Intermediate filament proteins of the liver: emerging disease association and functions. *Hepatology*, **25**, 1043–1048.

- Omary,M.B., Ku,N.-O., Liao, J. and Price,D. (1998) Keratin modifications and solubility properties in epithelial cells and *in vitro*. *Intermediate Filaments*, Subcellular Biochemistry Series, Plenum Publishing Co., NY, in press.
- Oshima,R.G., Millan,J.L. and Cecena,G. (1986) Comparison of mouse and human keratin 18: a component of intermediate filaments expressed prior to implantation. *Differentiation*, **33**, 61–68.
- Pallas,D.C., Fu,H., Haehnel,L.C., Weller,W., Collier,R.J. and Roberts,T.M. (1994) Association of polyomavirus middle tumor antigen with 14-3-3 proteins. *Science*, **265**, 535–537.
- Peng,C.-Y., Graves,P.R., Thoma,R.S., Wu,Z., Shaw,A.S. and Pinna-Worms,H. (1997) Mitotic and G₂ checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of cdc25c on serine-216. *Science*, **277**, 1501–1505.
- Reuther,G.W., Fu,H., Cripe,L.D., Collier,R.J. and Pendergast,A.M. (1994) Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science*, **266**, 129–133.
- Soellner,P., Quinlan,R.A. and Franke,W.W. (1985) Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. *Proc. Natl Acad. Sci. USA*, **82**, 7929–7933.
- Steinert,P.M. and Roop,D.R. (1988) Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.*, **57**, 593–625.
- Zha,J., Harada,H., Yang,E., Jockel,J. and Korsmeyer,S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X_L. *Cell*, **87**, 619–628.
- Zhang,S.-H., Kobayashi,R., Graves,P.R., Pinna-Worms,H. and Tonks,N.K. (1997) Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3 β protein. *J. Biol. Chem.*, **272**, 27281–27287.

*Received June 4, 1997; revised January 2, 1998;
accepted February 2, 1998*