Protein phosphatase-2A associates with and dephosphorylates keratin 8 after hyposmotic stress in a site- and cell-specific manner

Guo-Zhong Tao1,2,*, Diana M. Toivola1,2, Qin Zhou1,2, Pavel Strnad1,2, Baohui Xu2,3, Sara A. Michie2,3 and M. Bishr Omary1,2

1Department of Medicine, Palo Alto VA Medical Center, 3801 Miranda Avenue, Mail Code 154J, Palo Alto, CA 94304, USA
2Stanford University Digestive Disease Center, 300 Pasteur Drive, Stanford, CA 94305, USA
3Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA

*Author for correspondence (e-mail: guozhongtao@stanford.edu)

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Summary
Keratins 8 and 18 (K8 and K18) are regulated by site-specific phosphorylation in response to multiple stresses. We examined the effect and regulation of hyposmotic stress on keratin phosphorylation. K8 phospho-Ser431 (Ser431-P) becomes dephosphorylated in HT29 cells, but hyperphosphorylated on other K8 but not K18 sites in HRT18 and Caco2 cells and in normal human colonic ex vivo cultures. Hyposmosis-induced dephosphorylation involves K8 but not K18, K19 or K20, occurs preferentially in mitotically active cells, and peaks by 6-8 hours then returns to baseline by 12-16 hours. By contrast, hyperosmosis causes K8 Ser431 hyperphosphorylation in all tested cell lines. Hyposmosis-induced dephosphorylation of K8 Ser431-P is inhibited by okadaic acid but not by tautomycin or cyclosporine. The PP2A catalytic subunit co-immunoprecipitated with K8 and K18 after hyposmotic stress in HT29 cells, but not in HRT18 or Caco2 cells where K8 Ser431 becomes hyperphosphorylated. K8 Ser431-P dephosphorylation after hyposmosis was independent of PP2A levels but correlated with increased PP2A activity towards K8 Ser431-P. Therefore, hyposmotic stress alters K8 phosphorylation in a cell-dependent manner, and renders K8 Ser431-P a physiologic substrate for PP2A in HT29 cells as a result of PP2A activation and the physical association with K8 and K18. The divergent hyposmosis versus hyperosmosis K8 Ser431 phosphorylation changes in HT29 cells suggest that there are unique signaling responses to osmotic stress.

Key words: Intermediate filaments, Keratins 8 and 18, Phosphorylation, Dephosphorylation, Osmotic stress

Introduction
Intermediate filaments (IF) are a major component of the three cytoskeletal networks that include the actin microfilament and tubulin microtubule networks (Ku et al., 1999; Chang and Goldman, 2004). Compared with the limited number of actins and tubulins, IF proteins make up a large family of tissue- and differentiation-specific proteins that are encoded by more than 60 unique genes (Hesse et al., 2001; Herrmann and Aebi, 2004). Mutations of IF genes are associated with a variety of structural and nonstructural cellular functions, with the most prominent being the protection of epithelial cells from mechanical and nonmechanical stresses (Coulombe and Omary, 2002; Fuchs and Cleveland, 1998; Omary et al., 2004). Nonmechanical stresses, which have physiologic relevance to human disease pathogenesis, have been examined in the context of keratins and include a variety of toxins, osmotic or heat stress, and other agents that induce apoptosis. For example, in keratinocytes, naturally occurring mutations that cause epidermolysis bullosa simplex render these cells more susceptible to hyposmotic stress (D’Alessandro et al., 2002).

In cultured cell lines, hyposmotic stress induces cell-growth arrest followed by apoptosis or cell-cycle catastrophe, depending on the cell line, via proteosomal activation and subsequent degradation of a broad range of cell-cycle-related proteins (Tao et al., 2002).

An important aspect of keratin (and other IF protein) cytoprotective function regulation involves phosphorylation and interaction with keratin-associated proteins. For example, K8 and K18 phosphorylation increases during liver disease progression and appears to regress during recovery from liver injury (Toivola et al., 2004), and mutation of a major K18 phosphorylation site in transgenic mice predisposes them to...
liver injury (Ku et al., 1998b). In addition, K8 and K18 associate, in a highly regulated manner, with several binding partners including 14-3-3 proteins (Liao and Omary, 1996; Ku et al., 2002b), Raf-1 kinase (Ku et al., 2004) and the small GTP-binding protein Ran (Tao et al., 2005). In the case of K8, its major in vivo phosphorylation sites (Ser23, Ser73 and Ser431 for human K8) have been identified, and epitope-specific antibodies have been generated as an important tool to track keratin phosphorylation (Ku and Omary, 1997; Liao et al., 1997). Also, several probable in vivo K8 kinases have been identified including Erk1/2 for K8 Ser431 (Ku and Omary, 1997), and p38 and Jun kinases for K8 Ser73 (Ku et al., 2002a; He et al., 2002). However, no phosphatases are known to directly interact with and dephosphorylate K8 or K18, except for several previous studies showing that exposure of cells or tissues to phosphatase inhibitors causes dramatic hyperphosphorylation of K8 and K18 (Liao et al., 1995a; Favre et al., 1997; Toivola et al., 1997; Toivola et al., 2002). In addition, the importance of phosphatases in regulating intermediate-filament dynamics has been demonstrated for many IF proteins (Eriksson et al., 1992; Strnad et al., 2001) (reviewed by Ku et al., 1996; Eriksson et al., 1998). Protein phosphatase 2A (PP2A) is an essential cytoplasmic serine/threonine phosphatase involved in signal-transduction pathways that regulate a wide range of important cellular events including DNA replication, transcription, translation, growth and cell-cycle progression and cell transformation (Mumbiy and Walter, 1993; Wera and Hemmings, 1995; Chen et al., 2004). PP2A directly interacts with, and dephosphorylates, several important cellular proteins including Bcl2 (Deng et al., 1998) and Raf-1 kinase (Abraham et al., 2004).

In the present study, we show that hyposmosis results in site-specific dephosphorylation of K8 in HT29 cells by association with the catalytic subunit of PP2A, whereas hyperosmosis induces K8 hyperphosphorylation in the same cells. K8 dephosphorylation in HT29 cells after hypotonic exposure is cell-specific in that it does not occur in normal colon ex vivo cultures or in two other tested tumor cell lines. These findings suggest unique signaling responses to hyposmosis versus hyperosmosis and provide direct evidence for the regulation of K8 phosphorylation by PP2A.

**Results**

**Phosphorylation changes of HT29 cell K8 Ser431 in response to extracellular osmotic alterations**

We examined the effect of hyper- and hyposmosis changes on K8 and K18 phosphorylation using site-specific anti-phosphokeratin antibodies. As shown in Fig. 1A and in comparison with isotonic control conditions (lanes 1, 6), dramatic dephosphorylation of K8 Ser431-P was noted upon hyposmotic exposure for 6 hours (lane 3), which is reversible and returns to basal levels by 12 hours. By contrast, K8 Ser431 becomes hyperphosphorylated upon exposure to hypertonic conditions (Fig. 1A; lanes 8 and 9). On other hand, K8 Ser73, which becomes phosphorylated during mitosis and apoptosis and in response to a variety of stresses (Liao et al., 1997), was hyperphosphorylated after both hyposmotic (Fig. 1A, lanes 2-4) or hyperosmotic stress (Fig. 1A, lanes 7-9). These phosphorylation changes are selective in that two major K18 phosphorylation sites (Ser33 and Ser52) (Ku et al., 1998a) had no significant changes, regardless of cell exposure to hypo- or hyperosmotic conditions. As a positive control, we used heat shock, which induces keratin hyperphosphorylation (Fig. 1A, lanes 5 and 10) as reported previously (Liao et al., 1995b). Hence, the K8 Ser431-containing motif is unique in that hyposmosis may decrease or increase its phosphorylation in a stress-dependent manner.

**Fig. 1.** Effect of osmotic milieu changes on keratin phosphorylation: (A) HT29 cells were cultured at 37°C in isosmotic (lanes 1, 6; 12 hours), hyposmotic (lanes 2-4; 1, 6 or 12 hours, respectively) and hyperosmotic (lanes 7-9; 1, 6 or 12 hours, respectively) conditions. As a positive control for keratin hyperphosphorylation, cells were cultured at 42°C for 12 hours (lanes 5, 10). Total cell lysates were then prepared, transferred to PVDF membranes, followed by blouting with antibodies to the indicated epitopes (pS431, Ser431-P; pS73, Ser73-P; pS33, Ser33-P; pS52, Ser52-P). Asterisks highlight a unique K8 phosphorylated species (owing to K8 Ser73 phosphorylation) that has a slightly delayed migration in SDS-PAGE gels (Liao et al., 1997). Blooting with antibodies to total K8 or K18 is included to ensure equal protein loading. (B) HT29 cells were plated followed by exposure to isosmotic (I; lanes 1, 3, 5, 7) or hyposmotic (H; lanes 2, 4, 6, 8) conditions for 6 hours during log-phase (40-50% confluency) or subconfluent (70-80%) growth in the presence or absence of serum. Total cell lysates were subjected to immunoblot analysis using the indicated antibodies.
Keratin 8 dephosphorylation by PP2A occurs after exposure to epidermal growth factor (Ku and Omary, 1997), we tested the effect of serum and the cell cycle on hyposmosis-induced K8 dephosphorylation/phosphorylation (Fig. 1B). Serum depletion during the 6-hour culture period had no effect on K8 Ser431 phosphorylation in isotonic conditions (Fig. 1B, compare lanes 1 with 3 and lanes 5 with 7) but accentuates K8 Ser431 dephosphorylation (Fig. 1B, compare lanes 2 with 4 and lanes 8 with 6) which may be related to an EGF (or other growth factor) effect on promoting a relatively hyperphosphorylated Ser431 state. K8 Ser431 hyposmosis-induced dephosphorylation is more efficient in actively dividing cells, and so are the contrasting changes of K8 Ser73 hyperphosphorylation in response to serum depletion (Fig. 1B).

Therefore, HT29 cell K8 Ser431 undergoes divergent phosphorylation changes in response to hyposmotic versus hyperosmotic changes, and these phosphorylation changes are more dramatic in dividing rather than quiescent cells.

K8 dephosphorylation time course and analysis by 2D gels
K8 Ser431-P dephosphorylation in response to hypotonic conditions is not a rapid event but begins after 4 hours, peaks at 6 hours, then returns to basal levels by 12-16 hours (Fig. 2). By contrast, K8 Ser73 hyperphosphorylation in response to hypotonic conditions increases rapidly (within 1 hour) and is sustained up to 16 hours (Fig. 2). K18 appears to be less affected by hyposmosis, as demonstrated by the stable K18 Ser33 (Fig. 2) and K18 Ser52 phosphorylation (not shown). Hence, K8 dephosphorylation is reversible despite persistent exposure to hyposmotic conditions.

The overall changes in HT29 cell K8 and K18 phosphorylation in response to hyposmotic stress were also evaluated by IEF/SDS-PAGE 2-dimensional gel analysis. The three acidic K8 phospho-containing Coomassie-Blue-stained isofoms under isomotic conditions (isofoms 2-4) are recognized by anti-K8 Ser431-P antibody (Fig. 3a,c). Hyposmotic conditions significantly decrease anti-K8 Ser431-P antibody binding with commensurate decrease in the relative prominence of Coomassie-Blue-stained isofoms 2-4 (Fig. 3b,d). By contrast, the intensity of the two Coomassie-Blue-stained K18 isofoms is relatively unchanged (Fig. 3a,b). Other K8 site(s) remain phosphorylated after exposure to hypotonic conditions (e.g. isoform 2; Fig. 3b), which is probably related to the known phosphorylation of normal K8 at Ser23 under isomotic conditions (Ku and Omary, 1997).

We also tested the effect of hyposmosis on the phosphorylation state of K19 and K20 in HT29 cells. These two keratins are expressed at much lower levels compared with K8 and K18 (Fig. 4A). Phosphoepitope-specific antibody blot analysis of pK19 (at Ser35) and pK20 (at Ser13) showed that hyposmotic stress increases the phosphorylation of K20 Ser13-P (our unpublished observations) but not K19 Ser35-P (Fig. 4B) (Zhou et al., 1999), which are established phosphorylation sites. Total keratin protein levels were assessed using anti-K19 and anti-K20 antibodies, and blots of lysates isolated from okadaic acid (OA)-treated cells confirmed the phospho-specificity of the antibodies (Fig. 4B). Overall phosphorylation changes were assessed indirectly using 2D gel analysis (Fig. 4C), which showed that K19 phosphorylation did not significantly change (based on lack of acidic isoform shifting). By contrast, K20 phosphorylation did increase based on the appearance of isoform 5 and the relative increased intensity of isoform 4, which support the increase in K20 Ser13 phosphorylation (Fig. 4B). Hence, hyposmosis exposure in HT29 cells results in K8 dephosphorylation, K20...
hyperphosphorylation and no significant overall change in K18 or K19 phosphorylation (Figs 3 and 4).

**Effect of hyposmosis on K8 Ser431 phosphorylation in other human cell lines and in ex vivo colon cultures**

To address whether hyposmosis-induced dephosphorylation at Ser431 is a general cellular response, we tested the effect of hyposmosis stress on other human epithelial cell lines or human colon biopsies. In contrast to HT29 cells that respond to hyposmosis stress by K8 Ser431-dephosphorylation, HRT18 and Caco2 cells undergo K8 Ser431 hyperphosphorylation at these conditions (Fig. 5A). In the case of K8 Ser73, its phosphorylation behaves like (i.e. increases) its HT29 cell counterpart as does K18 Ser33 phosphorylation (i.e. no change in response to hyposmosis; Fig. 5A). Similarly, K8 Ser431 phosphorylation also increases in normal colon biopsies when cultured ex vivo under hyposmotic conditions (Fig. 5B, compare lanes 2 and 3). Notably, simple placement of the colon biopsies in culture induces K8 Ser431 phosphorylation (Fig. 6). A phosphorylation decrease in HT29 cells but an increase in Caco2 cells (Fig. 6). Another conclusion from the findings of Fig. 6 is that the phosphorylation changes are not clonal, in that most if not all of the cells are affected. Overall, these results indicate that HT29 cells have distinct mechanisms for regulating phosphorylation/dephosphorylation at Ser431 of K8.

PP2A, but not PP1 or PP2B, inhibitors induce K8 Ser431 hyperphosphorylation

There are many serine/threonine-specific protein phosphatases that are responsible for regulating the phosphorylation state of a wide range of proteins (Hunter, 1995). We examined the potential role of PP1, PP2A and PP2B in K8 Ser431-dephosphorylation using phosphatase-selective inhibitors. Tautomycin and cyclosporine A, which inhibit PP1 and PP2B respectively (Li and Casida, 1992; Liu et al., 1991), had no
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significant effect on HT29 cell K8 Ser431 phosphorylation (Fig. 7A, lanes 2,3). However, treatment with increasing concentrations of OA, which inhibits PP2A more efficiently than PP1, had a dramatic effect in HT29 cells (Fig. 7B, lanes 7-12) but a minimal effect in HRT18 cells (Fig. 7B, lanes 1-6) with regard to K8 Ser431 phosphorylation. Similar results to those obtained in HRT18 cells were also noted in Caco2 cells (not shown). The effect of OA in inducing HT29 cell K8 Ser431 hyperphosphorylation was more dramatic in hypotonic as compared with isosmotic conditions. As compared with K8 Ser431, the effect of OA on K8 Ser73 phosphorylation during hyposmosis in HRT18 cells was significant (Fig. 7B, compare lane 6 with lanes 4 and 5) but still markedly less than that noted in HT29 cells (Fig. 7B, lane 12) which supports the conclusion that PP2A activity is higher in HT29 as compared with HRT18 cells. Taken together, PP2A appears to be a potential physiologic phosphatase that is involved in K8 Ser431-P dephosphorylation.

PP2A associates with K8 and K18 in HT29 but not HRT18 cells after hyposmotic stress

The results shown in Fig. 7 suggest that HT29 cells have a higher PP2A activity compared with HRT18 cells. We tested and confirmed that the increased HT29 cell PP2A activity is not related to differences in the PP2A catalytic 36 kDa subunit levels between the two cell lines under basal or hyposmotic conditions (Fig. 8A). In addition, keratin and PP1 levels are similar when compared in HRT18 and HT29 cells (Fig. 8A). These overall findings led to test for a potential association of PP2A with keratins. Immunoprecipitation analysis revealed that the PP2A catalytic 36 kDa subunit associates with K8 and K18 only in HT29 cells and only during hyposmotic conditions (Fig. 8B). Therefore, the physical K8/K18-PP2A interaction explains the observed K8 Ser431-P dephosphorylation in HT29 cells but not in HRT18 cells during hyposmosis.

Discussion

The overall findings of the present study, which are summarized schematically in Fig. 9, demonstrate: (1) dramatic K8 Ser431-P dephosphorylation in HT29 cells upon exposure
to hyposmosis, whereas K8 Ser73 becomes hyperphosphorylated. This suggests that there are at least two independent phosphatases that regulate these two proline-directed-kinase phosphorylation sites (71LL and 429LT); (2) the four different keratins found in HT29 cells behave differently in terms of their phosphorylation response to hyposmotic stress. As such, dephosphorylation occurs only in K8 whereas hyperphosphorylation occurs in K20 and minimal, if any, changes occur in K18 and K19; (3) in contrast to findings in HT29 cells, hyposmosis exposure in Caco2 or HRT18 cells, and in normal colonic biopsies cultured ex vivo, results in K8 Ser431 hyperphosphorylation in association with blunted PP2A activity; (4) in contrast to hyposmosis, hyperosmosis induces K8 Ser431 hyperphosphorylation in all situations tested; (5) PP2A is probably a physiologic phosphatase that dephosphorylates K8 Ser431-P during hyposmotic conditions. PP2A may also serve as a phosphatase for other IF proteins including neurofilaments (Saito et al., 1995) and vimentin (Turowski et al., 1999), although the specific vimentin and neurofilament phosphorylation site(s) affected by PP2A are not known.

Ser431 is a major in vivo K8 phosphorylation site, which localizes at the K8 tail domain (Fig. 8). This site is conserved among mouse, rat and human K8 but is not found in other type II keratins, which suggests a unique function related to glandular epithelia (Ku and Omary, 1997). Limited information is known regarding specific phosphatases that may dephosphorylate K8 or other keratins, but several other studies previously showed that exposure of cells or tissues to phosphatase inhibitors causes dramatic hyperphosphorylation of K8 and K18 (Omary et al., 1996; Favre et al., 1997; Eriksson et al., 1998; Toivola et al., 2002). Keratin (and other IF protein) hyperphosphorylation occurs in association with most if not all injury situations that have been tested in cultured cells and animals, and in hepatocytes of patients with liver disease (Coulombe and Omary, 2002; Toivola et al., 2004). The study herein adds osmotic stress (both hypo and hyper) as inducers of increased phosphorylation of human K8 (at Ser73 and in some situations at Ser431), but not K18 Ser33/Ser52 (whose phosphorylation increases in response to most other stressors that have been tested), except for the dephosphorylation of K8 Ser431-P in response to hyposmosis that occurs in HT29 cells (Figs 1-3, 5).

Several lines of evidence support the conclusion that PP2A is a probable K8 Ser431-P physiologic phosphatase, including the use of selective inhibitors to PP2A, PP1 and PP2B (Fig. 7). Although OA inhibits PP1 and PP2A, its inhibition of PP2A is 50-100 times more sensitive than that of PP1 (Schonthal, 1998), and no induction of K8 Ser431 phosphorylation was noted after treatment of cells with the PP1 inhibitor tautomycin (Fig. 7). More importantly, the 36-kDa PP2A catalytic domain associates specifically with K8 and K18 immunoprecipitates during hyposmotic conditions (Fig. 8). The PP2A holoenzyme exists as ‘ABC’ heterotrimeric, composed of a core dimer of a 60-kDa ‘A’ subunit and a 36-kDa catalytic ‘C’ subunit, plus a variety of distinct regulatory ‘B’ subunits (Janssens and Goris, 2001). Regulation of PP2A activity can occur not only by subunit interactions, but also via casein kinase 2 (Heriche et al., 1997), methylation (Yu et al., 2001), or the SV40 small-t
antigen (Yang et al., 2005). It is likely that one or more of these or other regulatory mechanisms accounts for the observed differences in PP2A activity in HT29 cells versus HRT18 and Caco2 cells. For example, cultured breast MCF7 cells, in contrast to breast quiescent normal HBE cells, have significantly decreased PP2A activity owing to decreased levels of its catalytic subunit (Suzuki and Takahashi, 2003).

Although ex vivo cultures of normal human colonic biopsies behaved similarly to HRT18 and Caco2 cells in terms of hyperosmophorylation of K8 Ser431 after hyposmotic stress, it is noteworthy that simple culturing of the colonic biopsies ex vivo under isosmotic conditions induces dramatic hyperosmophorylation of K8 Ser431 (Fig. 4). We posit that the near-absent phosphorylation of K8 Ser431 when analyzed immediately after colonic biopsy removal suggests a high level of phosphatase activation under basal conditions, possibly related to PP2A, which becomes inactivated upon ex vivo culture of the biopsies. For example, culturing of normal pancreatic acinar cells away from their natural environment is a form of stress that activates stress-related kinases including p38 (Bliman et al., 2000). In support of this, inhibition of PP2A activates p38 kinase and Erk1/2 kinases in several tested model systems (Moriguchi et al., 1996; Lee et al., 2003; Ku et al., 2004). Another consideration for the potential significance of our findings is that mutations that play a regulatory role in PP2A-related pathways could be found in some epithelial tumors (given our finding in HT29 cells but not in HRT18, Caco2 or normal colon). This hypothesis can now be tested ex vivo on fresh tumor specimens using K8 Ser431 phosphorylation as a possible in vivo surrogate marker for PP2A-related pathways could be found in some epithelial tumors (Moriguchi et al., 1996; Lee et al., 2003; Ku et al., 2004).

The net observed phosphorylation state of a given phosphoepitope reflects the overall dual effects of kinases and phosphatases. A decrease in kinase activity and/or an increase in phosphatase activity could result in a net dephosphorylation state. A rapid (within 5-60 minutes) hyposmosis-induced activation of JNK and p38 kinases have been reported in primary hepatocytes, keratinocytes, HaCaT/A-431 cells (Kim et al., 2001; D’Alessandro et al., 2002; Kippenberger et al., 2005). In that regard, using specific antibodies to activated JNK and p38 to test their activities in HT29, Caco2 and HRT18 cells after 6 hours of hyposmotic exposure showed either an increase or basal kinase activation (not shown), thereby indicating that these kinases are unlikely to contribute to the specific dephosphorylation of Ser431-P in HT29 cells. This, coupled with the observations related to the phosphatase-related experiments summarized above lend support to our conclusion that K8 Ser431-P is a physiologic substrate to PP2A during hyposmotic conditions and possibly other biologic contexts.

Materials and Methods

Regents, cell culture and hyposmotic treatment

Immunobilized protein-A Sepharose (Zymed Laboratories, South San Francisco, CA), BCA protein assay kit (Pierce, Rockford, IL), and antibodies (Ab) to the catalytic subunit of protein phosphatase PP1 and PP2A (BD Biosciences, San Diego, CA) were used. Three human colonic cell lines Caco2, HRT18, HT29 (American Type Culture Collection, Manassas, VA) were cultured as recommended by the supplier. Hyposmotic treatment was carried out as described (Tao et al., 2002), and hyperosmotic treatment was performed by exposing cells to normal culture medium mixed (1:1, v/v) with 0.45 M NaCl in water. As a control, cells were also cultured in iso-osmotic medium prepared by diluting each medium with equal volume of water containing 0.15 M sodium chloride (Zhang et al., 1998).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Cells grown on plates were rapidly rinsed with pre-warmed PBS containing 5 mM EDTA, scraped into collection tubes, then solubilized in hot SDS-PAGE sample buffer. The solubilized cells were separated by SDS-PAGE (Laemmli, 1970) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) followed by immunoblotting (Towbin et al., 1979). The antibodies used included those directed to: human total K8 (M20) or K18 (DC10) (NeoMarkers, Fremont, CA); phosphokerkatins K8 pS73 (Ser73-P) (LJ4) (Liao et al., 1997) or pS431 (Ser431-P) (SB3; NeoMarkers, Fremont, CA) (Ku and Omary, 1997), K18 pS33 (Ser33-P) (Ab 8250) (Ku et al., 1998a) or pS52 (Ser52-P) (Ab 3055; Liao et al., 1995a), K9 pS53 (Ser53-P) (Zhou et al., 1999) and K20 pS13 (Ser13-P) (our unpublished observations). Immunoblotted proteins were visualized using enhanced chemiluminescence as recommended by the supplier (NEN TM Life Science Products, Boston, MA).

Immunoprecipitation and 2D gel electrophoresis

Cells were solubilized (60 minutes, 4°C) in 1% Empigen BB (Emp) in PBS containing 0.1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, 10 µM leupeptin, 10 µM pepstatin, 5 nM EDTA, and 0.5 µg/ml okadaic acid. After pelleting (16,000g for 30 minutes at 4°C), the solubilized material was incubated with anti-K8/K18 Ab L2A1 (Chou et al., 1992) (NeoMarkers, Fremont, CA) that was covalently conjugated to protein A-Sepharose beads (2 hours, 4°C). The immune complex-containing beads were washed then analyzed by SDS-PAGE, or by 2D separation using isoelectric focusing (IEF) in the first dimension followed by SDS-PAGE in the second dimension (Liao et al., 1996).

Immunofluorescence cell staining

Cells were grown on multi-chamber slides then fixed with pre-cooled methanol (~20°C, 10 minutes) after washing using pre-warmed PBS. Fixed cells were double-stained with anti-K8/K18 rabbit Ab 8592 (Ku and Omary, 1997), which recognizes total K8 and K18, and with anti-Ser431-P mouse Ab 383 (Ku and Omary, 1997). Staining was visualized with Texas Red-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) and FITC-conjugated goat anti-mouse (Biosourse, Camarillo, CA) secondary antibodies followed by confocal microscopy. For HT29 cells, nuclei were stained with TOTO-3 iodide (Molecular Probes, Eugene, OR).

Treatment with protein phosphatase inhibitors

Cells were incubated with okadaic acid (100 and 625 nM) (LC Services, Woburn, MA), tautomycin (625 nM) (Alexis Biochemicals, San Diego, CA) or cyclosporine A (1 µg/ml) (Sigma, St Louis, MO) (Woeckmann et al., 1999) at 37°C for the indicated periods.

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