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

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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 wide spectrum of diseases that reflect their tissue-selective expression (Fuchs and Cleveland, 1998; Omary et al., 2004). IF proteins include the nuclear lamins that typically link with the inner nuclear membrane, and a large group of cell-specific cytoplasmic members that can be visualized by immunofluorescence staining as filamentous arrays extending from the perinuclear region to the plasma membrane. In epithelial cells, the major IF proteins consist of keratins (K) which include type I (K9-K20, relatively acidic) and type II (K1-K8, neutral or basic) IF (Coulombe and Omary, 2002). All epithelial cells express at least one type I and one type II keratin that associate as obligate heteropolymers in a noncovalent fashion.

In simple-type epithelia, as found in liver, pancreas and gastrointestinal tract, K8 and K18 are the major keratins with additional variable levels of K19 and K20 depending on the cell type (Moll et al., 1982; Zhou et al., 2003). Keratins carry out 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 (Mumby 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., 2000).

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 hyposmotic 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

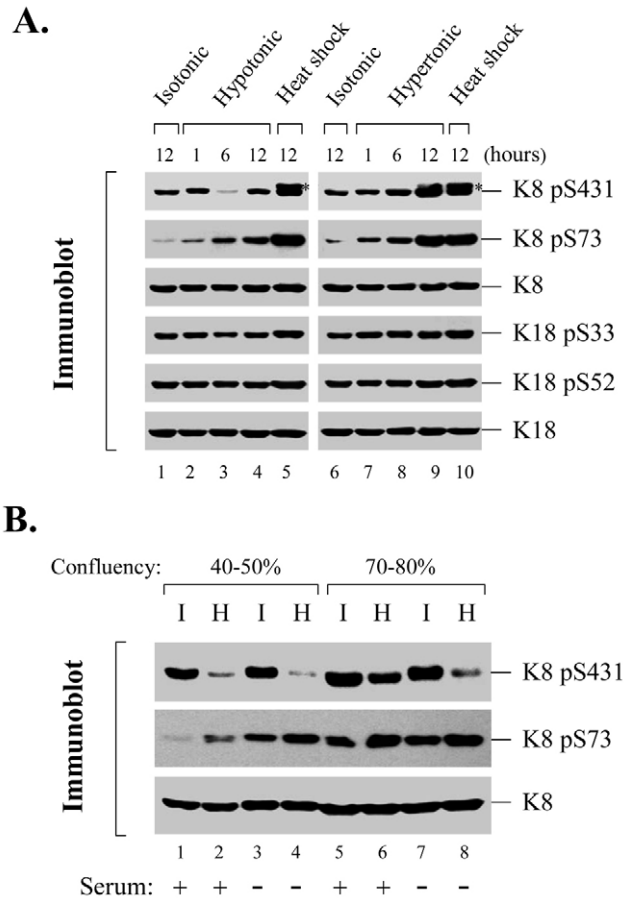

Fig. 1. Effect of osmotic milieu changes on keratin phosphorylation: (A) HT29 cells were cultured at 37°C in isosmotic (lanes 1,6; 12 hours), hyposmotic (lanes 2-4; 1,6 or 12 hours, respectively) and hyperosmotic (lanes 7-9; 1, 6 or 12 hours, respectively) conditions. As a positive control for keratin 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 blotting 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). Blotting 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.

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.

Hyposmotic stress of HT29 cells and other human colonic cell lines, including HRT18 and Caco2 (for a similar 6-hour exposure), causes cell-growth arrest (Tao et al., 2002). Given this observation and that K8 Ser431 hyperphosphorylation

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 4 with 2 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 isoforms under isosmotic conditions (isoforms 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 isoforms 2-4 (Fig. 3b,d). By contrast, the intensity of the two Coomassie-Blue-stained K18 isoforms 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 isosmotic 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

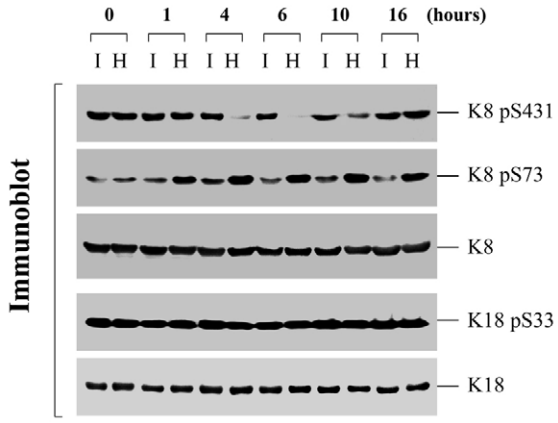

Fig. 2. Time course of K8 and K18 phosphorylation changes after hyposmotic exposure. HT29 cells were cultured in isosmotic (I) or hyposmotic (H) conditions (37°C) for the indicated time periods. Total cell lysates were prepared by mixing the cells with hot Laemmli sample buffer, followed by SDS-PAGE then immunoblotting using anti-phospho-keratin or anti-total-keratin antibodies as indicated.

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

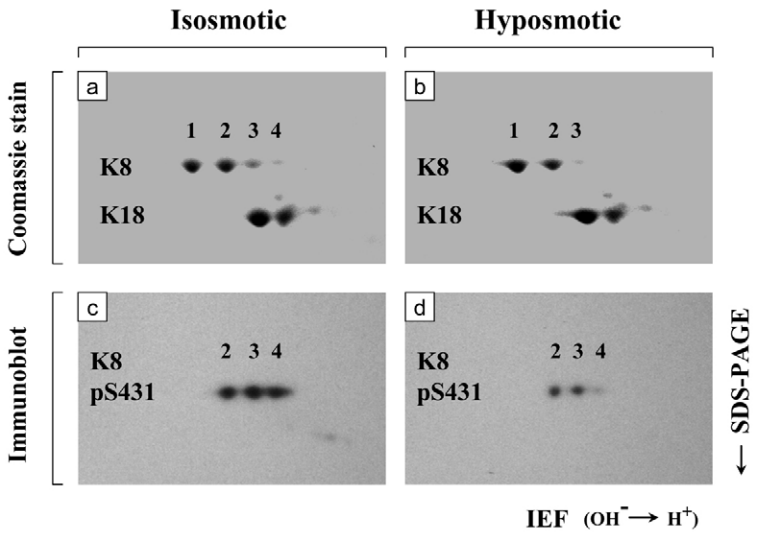

Fig. 3. Analysis of K8 phosphorylation and dephosphorylation by two-dimensional gel electrophoresis. HT29 cells were cultured in isosmotic or hyposmotic conditions for 6 hours, solubilized by 1% Emp in PBS (4°C, 60 minutes), followed by immunoprecipitation of K8 and K18. Precipitates were analyzed in duplicate by isoelectric focusing (IEF) in the first dimension followed by SDS-PAGE in the second dimension. One gel was then stained with Coomassie Blue and the second was transferred to a PVDF membrane for immunoblot analysis using anti-K8 Ser431-*P* antibodies. After blotting analysis, the PVDF membrane was stained with Coomassie Blue (not shown) in order to assign the indicated K8 charged isoforms.

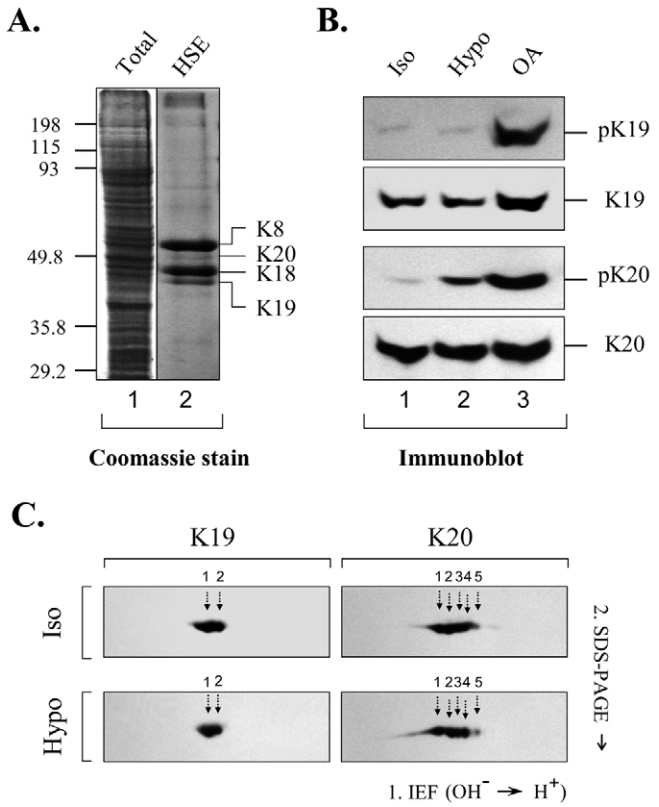

Fig. 4. Effect of hyposmosis on K19 and K20 phosphorylation. (A) Total cell extracts were prepared from HT29 cells (Total) or high salt extraction (HSE) was used to generate a highly enriched keratin fraction followed by analysis with SDS-PAGE and Coomassie Blue staining. (B) HT29 cells were incubated in isotonic or hypotonic medium for 6 hours, harvested, then subjected to SDS-PAGE followed by immunoblotting using antibodies to K19 or K20, or phospho-K19 or phosho-K20 (pK19 and pK20). Lanes 3 included homogenates isolated from OA-treated HT29 cells, as a positive control for the phospho-antibody reactivity. (C) Total cell lysates from iso- or hypo-treated HT29 cells (as in panel B) were analyzed by isoelectric focusing (IEF) in the first dimension followed by SDS-PAGE in second dimension then immunoblotting using anti-K19 or anti-K20 antibodies. Spot 1 is the non-phosphorylated isoform; others are phosphorylated isoforms for K19 and K20.

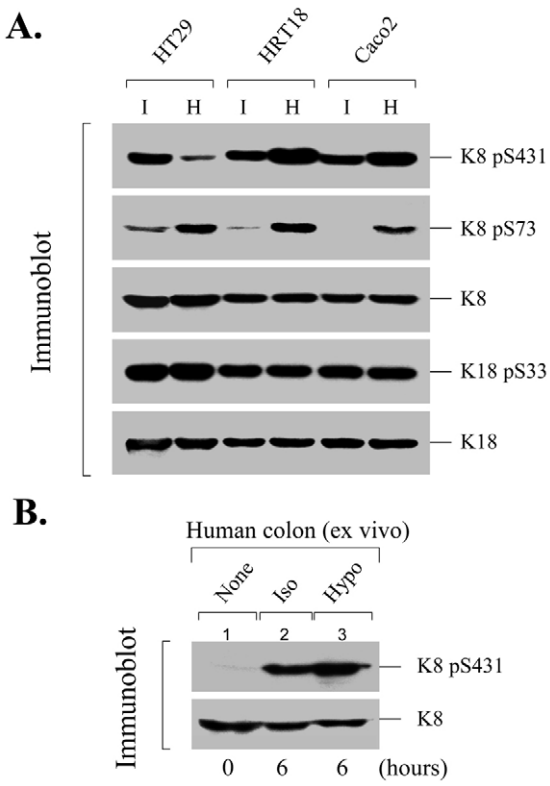

Fig. 5. Effect of hyposmotic stress on K8 and K18 phosphorylation in human colonic cell lines and ex-vivo-cultured colonic biopsies. (A) Three human colonic cell lines (HT29, HRT18 and Caco2) were cultured in isosmotic (I) or hyposmotic (H) medium at 37°C for 6 hours. Total cell lysates were blotted with antibodies to the indicated antigens. (B) Total tissue lysates were generated from normal human colon biopsies immediately upon isolation (none), or after culturing in isosmotic (Iso) or hyposmotic (Hypo) conditions (37°C, 6 hours). Lysates were then blotted with antibodies to total K8 or to K8 Ser431-*P*.

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 hyposmotic stress on other human colonic cell lines or human colon biopsies. In contrast to HT29 cells that respond to hyposmotic stress by K8 Ser431-*P* 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 colonic biopsies in culture induces K8 Ser431 phosphorylation (Fig. 5B, compare lanes 1 and 2). The differences in K8 Ser431 phosphorylation changes in HT29 versus HRT18 (not shown) and Caco2 cells were confirmed by immunofluorescence staining (i.e. 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-*P* 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

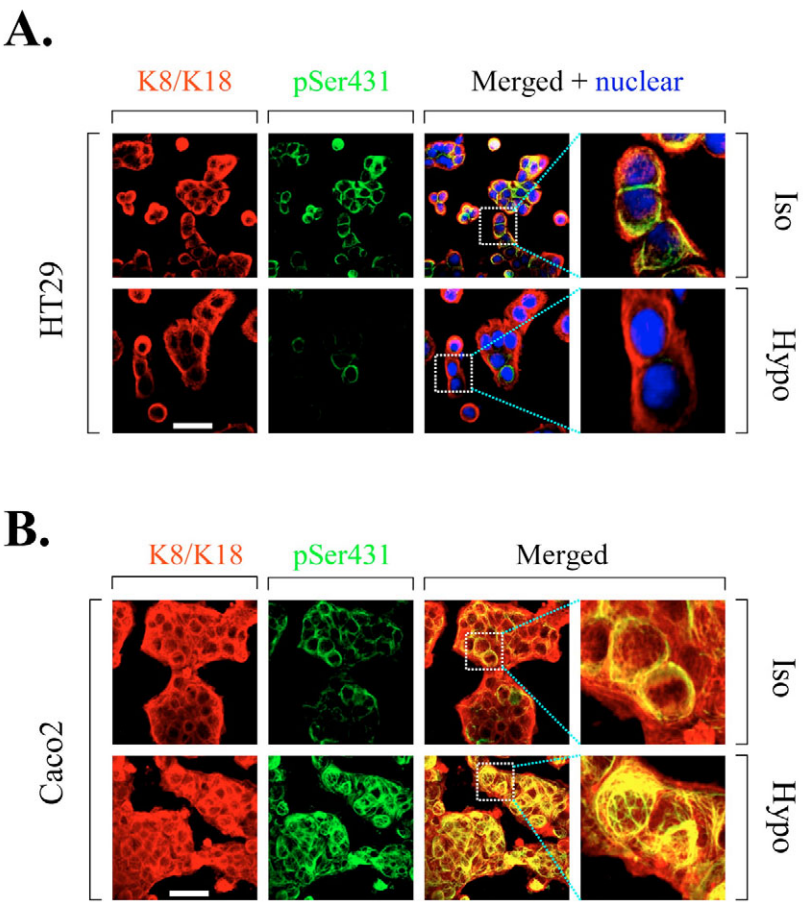

Fig. 6. Immunofluorescence analysis of keratins and phospho-keratins under isosmotic and hyposmotic conditions. HT29 (A) and Caco2 (B) cells were cultured in isosmotic (Iso) or hyposmotic (Hypo) conditions (6 hours, 37°C), then fixed and triple (K8/K18, K8 Ser431-*P* and nuclei in HT29 cells) or double (K8/K18, K8 Ser431-*P* in Caco2 cells) stained. The panels on the right are enlargements of the outlined 'boxed' areas. Bars, 25 μm.

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

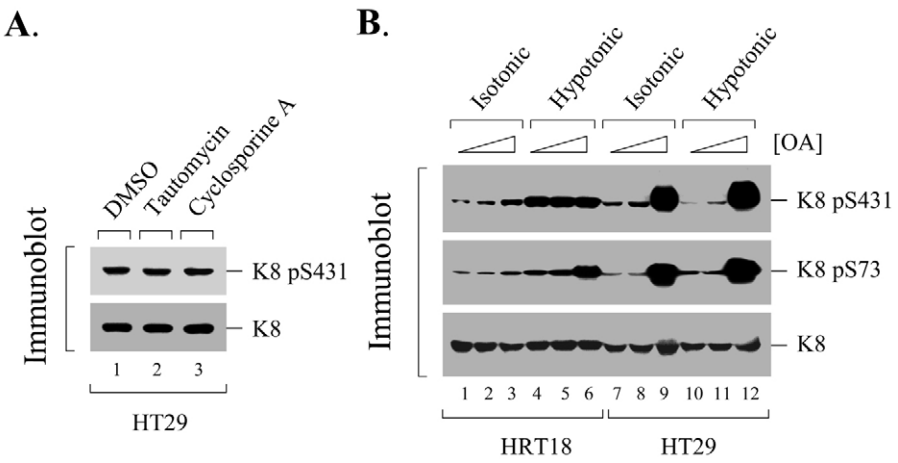

Fig. 7. Effect of protein phosphatase inhibition on K8 Ser73 and Ser431 phosphorylation. (A) HT29 cells, cultured under isosmotic conditions, were treated with tautomycin, cyclosporine-A or carrier control (DMSO) for 1 hour (37°C). Total cell lysates were then prepared and blotted with antibodies to total K8 or K8 Ser431-*P*. (B) HRT18 and HT29 cells were cultured in isosmotic or hyposmotic conditions for 5 hours, then DMSO (vehicle control; lanes 1, 4, 7, 10) or OA (100, 625 nM) were added to the culture media (1 hour). Total cell lysates were then prepared followed by immunoblotting.

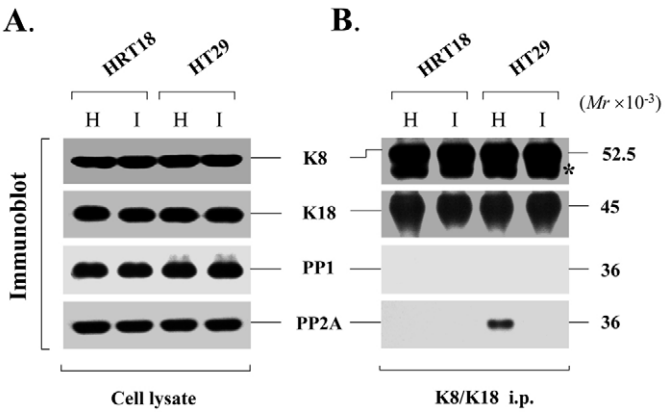

Fig. 8. Association of PP2A with K8 and K18 in HT29 cells after hyposmotic stress. HRT18 and HT29 cells were cultured in isosmotic (I) or hyposmotic (H) conditions (6 hours, 37°C), then solubilized with 1% NP40 in PBS (4°C). (A) The lysates were then analyzed by blotting using antibodies to K8, K18, PP1 or PP2A. (B) Alternatively, the NP40 lysates were used to immunoprecipitate K8 and K18 followed by blotting using the indicated antibody. Asterisk indicates the antibody heavy chain band.

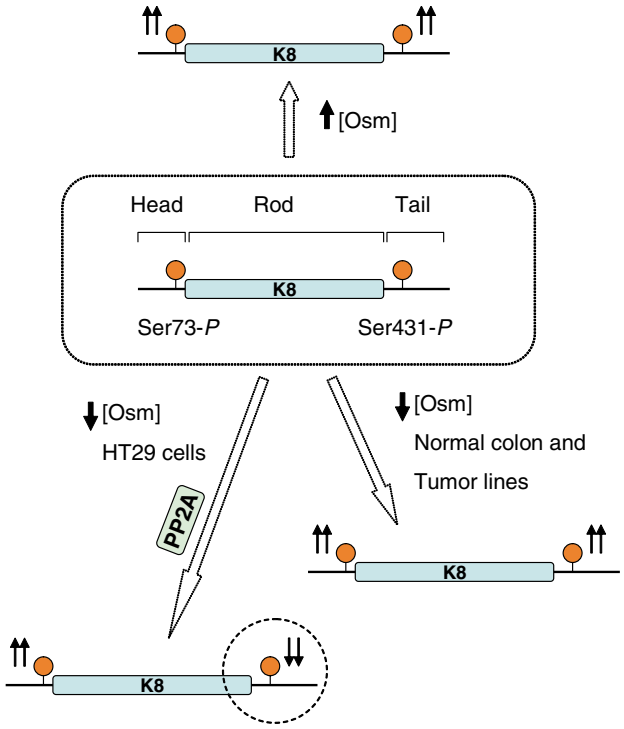

Fig. 9. Schematic of K8 phosphorylation changes in response to osmotic alterations. K8 (and all other IF proteins) consist of a central, relatively conserved, coiled-coil α-helical 'rod' domain that is flanked by non-α-helical relatively non-conserved 'head' and 'tail' domains (Herrmann and Aebi, 2004). The head and tail domains contain most of the known posttranslational modification of IF proteins (Coulombe and Omary, 2002; Omary et al., 2004). Hyperosmotic stress results in human K8 hyperphosphorylation (upward-pointing double arrows) at Ser73 and Ser431. Hyposmotic stress (downward-pointing double arrows) also induces K8 hyperphosphorylation at Ser73 and Ser431 except in the case of HT29 cells where it induces K8 dephosphorylation via PP2A.

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 (^{71}LL**S**PL and ^{429}LT**S**PG); (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' heterotrimers, 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 hyperphosphorylation of K8 Ser431 after hyposmotic stress, it is noteworthy that simple culturing of the colonic biopsies ex vivo under isosmotic conditions induces dramatic hyperphosphorylation 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 (Blinman 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 activity. Interestingly, a genetic variant of the Aβ scaffolding subunit of PP2A is seen in much higher frequency in breast cancer patients (3%) compared with controls (0.3%) (Esplin et al., 2005).

The net observed phosphorylation state of a given phospho-epitope 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 hyposmosis 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

Immobilized 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) then 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); phosphokeratins K8 pS73 (Ser73-*P*) (LJ4) (Liao et al., 1997) or pS431 (Ser431-*P*) (5B3; NeoMarkers, Freemont, 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), K19 pS35 (Ser35-*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™ 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 mM EDTA, and 0.5 μg/ml okadaic acid. After pelleting (16,000 ***g*** for 30 minutes at 4°C), the solubilized material was incubated with anti-K8/K18 Ab L2A1 (Chou et al., 1992) (NeoMarkers, Freemont, 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 5B3 (Ku and Omary, 1997). Staining was visualized with Texas Red-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) and FITC-conjugated goat anti-mouse (Biosource, 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 (400 ng/ml) (Sigma, St Louis, MO) (Woetmann et al., 1999) at 37°C for the indicated periods.

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References

Abraham, D., Podar, K., Pacher, M., Kubicek, M., Welzel, N., Hemmings, B. A., Dilworth, S. M., Mischak, H., Kolch, W. and Baccarini, M. (2000). Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. *J. Biol. Chem.* **275**, 22300-22304.

Blinman, T. A., Gukovsky, I., Mouria, M., Zaninovic, V., Livingston, E., Pandol, S. J. and Gukovskaya, A. S. (2000). Activation of pancreatic acinar cells on isolation from tissue: cytokine upregulation via p38 MAP kinase. *Am. J. Physiol. Cell Physiol.* **279**, C1993-C2003.

Chang, L. and Goldman, R. D. (2004). Intermediate filaments mediate cytoskeletal crosstalk. *Nat. Rev. Mol. Cell. Biol.* **5**, 601-613.

Chen, W., Possemato, R., Campbell, K. T., Plattner, C. A., Pallas, D. C. and Hahn, W. C. (2004). Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* **5**, 127-136.

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.

Coulombe, P. A. and Omary, M. B. (2002). 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol.* **14**, 110-122.

D'Alessandro, M., Russell, D., Morley, S. M., Davies, A. M. and Lane, E. B. (2002). Keratin mutations of epidermolysis bullosa simplex alter the kinetics of stress response to osmotic shock. *J. Cell Sci.* **115**, 4341-4351.

Deng, X., Ito, T., Carr, B., Mumby, M. and May, W. S., Jr (1998). Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. *J. Biol. Chem.* **273**, 34157-34163.

Eriksson, J. E., Brautigan, D. L., Vallee, R., Olmsted, J., Fujiki, H. and Goldman, R. D. (1992). Cytoskeletal integrity in interphase cells requires protein phosphatase activity. *Proc. Natl. Acad. Sci. USA* **89**, 11093-11097.

Eriksson, J. E., Toivola, D. M., Sahlgren, C., Mikhailov, A. and Harmala-Brasken, A. S. (1998). Strategies to assess phosphoprotein phosphatase and protein kinase-mediated regulation of the cytoskeleton. *Methods Enzymol.* **298**, 542-569.

Esplin, E. D., Ramos, P., Martinez, B., Tomlinson, G. E., Mumby, M. C. and Evans, G. A. (2005). The glycine 90 to aspartate alteration in the Abeta subunit of PP2A (PPP2R1B) associates with breast cancer and causes a deficit in protein function. *Genes Chromosomes Cancer* **45**, 182-190.

Favre, B., Turowski, P. and Hemmings, B. A. (1997). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. *J. Biol. Chem.* **272**, 13856-13863.

Fuchs, E. and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-519.

He, T., Stepulak, A., Holmstrom, T. H., Omary, M. B. and Eriksson, J. E. (2002). The intermediate filament protein keratin 8 is a novel cytoplasmic substrate for c-Jun N-terminal kinase. *J. Biol. Chem.* **277**, 10767-10774.

Heriche, J. K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E. M. and Goldberg, Y. (1997). Regulation of protein phosphatase 2A by direct interaction with casein kinase 2alpha. *Science* **276**, 952-955.

Herrmann, H. and Aebi, U. (2004). Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular Scaffolds. *Annu. Rev. Biochem.* **73**, 749-789.

Hesse, M., Magin, T. M. and Weber, K. (2001). Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J. Cell Sci.* **114**, 2569-2575.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236.

Janssens, V. and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417-439.

Kim, R. D., Darling, C. E., Roth, T. P., Ricciardi, R. and Chari, R. S. (2001). Activator protein 1 activation following hypoosmotic stress in HepG2 cells is actin cytoskeleton dependent. *J. Surg. Res.* **100**, 176-182.

Kippenberger, S., Loitsch, S., Guschel, M., Muller, J., Kaufmann, R. and Bernd, A. (2005). Hypotonic stress induces E-cadherin expression in cultured human keratinocytes. *FEBS Lett.* **579**, 207-214.

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., Liao, J., Chou, C. F. and Omary, M. B. (1996). Implications of intermediate filament protein phosphorylation. *Cancer Metastasis Rev.* **15**, 15429-15444.

Ku, N. O., Liao, J. and Omary, M. B. (1998a). Phosphorylation of human keratin 18 serine 33 regulates binding to 14-3-3 proteins. *EMBO J.* **17**, 1892-1906.

Ku, N. O., Michie, S. A., Soetikno, R. M., Resurreccion, E. Z., Broome, R. L. and Omary, M. B. (1998b). Mutation of a major keratin phosphorylation site predisposes to hepatotoxic injury in transgenic mice. *J. Cell Biol.* **143**, 2023-2032.

Ku, N. O., Zhou, X., Toivola, D. M. and Omary, M. B. (1999). The cytoskeleton of digestive epithelia in health and disease. *Am. J. Physiol.* **277**, G1108-G1137.

Ku, N. O., Azhar, S. and Omary, M. B. (2002a). Keratin 8 phosphorylation by p38 kinase regulates cellular keratin filament reorganization: modulation by a keratin 1-like disease causing mutation. *J. Biol. Chem.* **277**, 10775-10782.

Ku, N. O., Michie, S., Resurreccion, E. Z., Broome, R. L. and Omary, M. B. (2002b). Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression. *Proc. Natl. Acad. Sci. USA* **99**, 4373-4378.

Ku, N. O., Fu, H. and Omary, M. B. (2004). Raf-1 activation disrupts its binding to keratins during cell stress. *J. Cell Biol.* **166**, 479-485.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, T., Kim, S. J. and Sumpio, B. E. (2003). Role of PP2A in the regulation of p38 MAPK activation in bovine aortic endothelial cells exposed to cyclic strain. *J. Cell Physiol.* **194**, 349-355.

Li, Y. M. and Casida, J. E. (1992). Cantharidin-binding protein: identification as protein phosphatase 2A. *Proc. Natl. Acad. Sci. USA* **89**, 11867-11870.

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**, 345-357.

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., 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 Ser-73 in tissues and cultured cells. *J. Biol. Chem.* **272**, 17565-17573.

Liu, J., Farmer, J. D., Jr, Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815.

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.

Moriguchi, T., Toyoshima, F., Gotoh, Y., Iwamatsu, A., Irie, K., Mori, E., Kuroyanagi, N., Hagiwara, M., Matsumoto, K. and Nishida, E. (1996). Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H2O2. *J. Biol. Chem.* **271**, 26981-26988.

Mumby, M. C. and Walter, G. (1993). Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. *Physiol. Rev.* **73**, 673-699.

Omary, M. B., Coulombe, P. A. and McLean, W. H. (2004). Intermediate filament proteins and their associated diseases. *N. Engl. J. Med.* **351**, 2087-2100.

Saito, T., Shima, H., Osawa, Y., Nagao, M., Hemmings, B. A., Kishimoto, T. and Hisanaga, S. (1995). Neurofilament-associated protein phosphatase 2A: its possible role in preserving neurofilaments in filamentous states. *Biochemistry* **34**, 7376-7384.

Schonthal, A. H. (1998). Role of PP2A in intracellular signal transduction pathways. *Front. Biosci.* **3**, D1262-D1273.

Strnad, P., Windoffer, R. and Leube, R. E. (2001). In vivo detection of cytokeratin filament network breakdown in cells treated with the phosphatase inhibitor okadaic acid. *Cell Tissue Res.* **306**, 277-293.

Suzuki, K. and Takahashi, K. (2003). Reduced expression of the regulatory A subunit of serine/threonine protein phosphatase 2A in human breast cancer MCF-7 cells. *Int. J. Oncol.* **23**, 1263-1268.

Tao, G. Z., Rott, L. S., Lowe, A. W. and Omary, M. B. (2002). Hyposmotic stress induces cell growth arrest via proteasome activation and cyclin/cyclin-dependent kinase degradation. *J. Biol. Chem.* **277**, 19295-19303.

Tao, G. Z., Zhou, Q., Strnad, P., Salemi, M. R., Lee, Y. M. and Omary, M. B. (2005). Human Ran cysteine 112 oxidation by pervanadate regulates its binding to keratins. *J. Biol. Chem.* **280**, 12162-12167.

Toivola, D. M., Goldman, R. D., Garrod, D. R. and Eriksson, J. E. (1997). Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments. *J. Cell Sci.* **110**, 23-33.

Toivola, D. M., Zhou, Q., English, L. S. and Omary, M. B. (2002). Type II keratins are phosphorylated on a unique motif during stress and mitosis in tissues and cultured cells. *Mol. Biol. Cell* **13**, 1857-1870.

Toivola, D. M., Ku, N. O., Resurreccion, E. Z., Nelson, D. R., Wright, T. L. and Omary, M. B. (2004). Keratin 8 and 18 hyperphosphorylation is a marker of progression of human liver disease. *Hepatology* **40**, 459-466.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Turowski, P., Myles, T., Hemmings, B. A., Fernandez, A. and Lamb, N. J. (1999). Vimentin dephosphorylation by protein phosphatase 2A is modulated by the targeting subunit B55. *Mol. Biol. Cell* **10**, 1997-2015.

Wera, S. and Hemmings, B. A. (1995). Serine/threonine protein phosphatases. *Biochem. J.* **311**, 17-29.

Woetmann, A., Nielsen, M., Christensen, S. T., Brockdorff, J., Kaltoft, K., Engel, A. M., Skov, S., Brender, C., Geisler, C., Svejgaard, A. et al. (1999). Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. *Proc. Natl. Acad. Sci. USA* **96**, 10620-10625.

Yang, C. S., Vitto, M. J., Busby, S. A., Garcia, B. A., Kesler, C. T., Gioeli, D., Shabanowitz, J., Hunt, D. F., Rundell, K., Brautigan, D. L. et al. (2005). Simian virus 40 small t antigen mediates conformation-dependent transfer of protein phosphatase 2A onto the androgen receptor. *Mol. Cell Biol.* **25**, 1298-1308.

Yu, X. X., Du, X., Moreno, C. S., Green, R. E., Ogris, E., Feng, Q., Chou, L., McQuoid, M. J. and Pallas, D. C. (2001). Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. *Mol. Biol. Cell* **12**, 185-199.

Zhang, Z., Yang, X. Y. and Cohen, D. M. (1998). Hypotonicity activates transcription through ERK-dependent and -independent pathways in renal cells. *Am. J. Physiol.* **275**, C1104-C1112.

Zhou, X., Liao, J., Hu, L., Feng, L. and Omary, M. B. (1999). Characterization of the major physiologic phosphorylation site of human keratin 19 and its role in filament organization. *J. Biol. Chem.* **274**, 2861-2866.

Zhou, Q., Toivola, D. M., Feng, N., Greenberg, H. B., Franke, W. W. and Omary, M. B. (2003). Keratin 20 helps maintain intermediate filament organization in intestinal epithelia. *Mol. Biol. Cell* **14**, 2959-2971.