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Raf-1 activation disrupts its binding to keratins during cell stress

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Keratins 8 and 18 (K8/18) heteropolymers may regulate cell signaling via the known K18 association with 14-3-3 proteins and 14-3-3 association with Raf-1 kinase. We characterized Raf–keratin–14-3-3 associations and show that Raf associates directly with K8, independent of Raf kinase activity or Ras–Raf interaction, and that K18 is a Raf physiologic substrate. Raf activation during oxidative and toxin exposure in cultured cells and animals disrupt keratin–Raf association in a phosphorylation-dependent manner. Mutational analysis showed that 14-3-3 residues that are essential for Raf binding also regulate 14-3-3–keratin association. Similarly, Raf phosphorylation sites that are important for binding to 14-3-3 are also essential for Raf binding to K8/18. Therefore, keratins may modulate some aspects of Raf signaling under basal conditions via sequestration by K8, akin to Raf–14-3-3 binding. Keratin-bound Raf kinase is released upon Raf hyperphosphorylation and activation during oxidative and other stresses.

Introduction
Keratins are intermediate filament proteins that are specifically expressed in epithelial cells, and consist of noncovalent obligate associations of one or more type I (K9-20) and type II (K1-8) keratins in an epithelial-specific manner (Moll et al., 1982; Fuchs and Weber, 1994; Coulombe and Omary, 2002). Glandular or "simple-type" epithelial cells express preferentially the keratin pair K8/18, as cytoplasmic filamentous/oligomeric or soluble tetrameric heteropolymers that consist of two K8 and two K18 molecules (Quinlan et al., 1984; Omary et al., 1998). The best-characterized keratin function is to protect epithelial cells from necrotic or apoptotic forms of injury that are induced by mechanical and nonmechanical stresses (Gilbert et al., 2001; Coulombe and Omary, 2002; Oshima, 2002). This function is supported by numerous animal model studies and by the phenotypes of several keratin mutation-associated human diseases (Fuchs and Cleveland, 1998; Irvine and McLean, 1999; Magin et al., 2000). The cytoprotective and other proposed keratin functions are likely to be regulated by keratin phosphorylation and keratin-associated proteins (Coulombe and Omary, 2002). For example, K18 serine-33 (S33) phosphorylation regulates keratin binding to 14-3-3 proteins during mitosis or after exposure to phosphatase inhibitors in the context of intact tissues or cultured cells (Liao and Omary, 1996; Ku et al., 1998, 2002). The significance of keratin–14-3-3 interaction is related to the phosphorylation-dependent association of 14-3-3 proteins with a wide range of signaling molecules including Raf-1 kinase, the pro-apoptotic protein Bad, and cdc25 phosphatase among others (Fu et al., 2000; Tzivion and Avruch, 2002; Yaffe, 2002). Direct or indirect keratin–Raf interaction is not known but given the established associations of Raf kinase with 14-3-3 proteins and K8/18 with 14-3-3 proteins, we sought to define the dynamics, significance, and molecular domains that define Raf–keratin–14-3-3 associations.

Results and discussion
We examined the interrelationship of the known keratin–14-3-3 association (Liao and Omary, 1996; Ku et al., 1998) with the potential for a keratin–Raf association, in human colonic HT29 cells, given the established Raf–14-3-3 interaction (Tzivion et al., 1998). K8/18/19 immunoprecipitates were obtained from cells, with or without pretreatment with the phosphatase inhibitor okadaic acid (OA), followed by immunoblotting with antibodies (Abs) to Raf or 14-3-3 proteins. 14-3-3 binding to K8/18 increased dramatically due to K18 S33 hyperphosphorylation (Ku et al., 1998), but surprisingly Raf kinase associated with K8/18 under basal...
conditions and this association was abolished by phosphatase inhibition (Fig. 1 A, lanes 1 and 2). Raf–keratin association was also noted using other antikeratin Abs (anti-K19; Fig. 1 B), thereby indicating that it is unrelated to cross-reaction of antikeratin Abs with Raf. The keratin–Raf interaction was also abolished in mice upon intraperitoneal administration of the phosphatase inhibitor and hepatotoxin microcystin-LR (MLR; Fig. 1 A, lanes 3–6).

To distinguish between Raf binding to K8 versus K18 or K19, we transfected BHK cells (which lack keratins) with Raf, K8/18, K8/19, Raf/H11001K8/18, or Raf/H11001K8/19 followed by immunoprecipitation of K8/18 or K8/19. As shown in Fig. 1 B, transfected human Raf associates specifically with K8/18 or K8/19 (lanes 4 and 8), thereby indicating that K8 is necessary for keratin–Raf binding. Endogenous hamster Raf does not bind efficiently to the overexpressed keratins (Fig. 1 B, lanes 3 and 7). In vitro overlay assay confirmed that Raf interacts with K8, but not K18 (Fig. 1 C). Because hsp70 also associates with K8, we asked whether hsp70 is necessary for keratin–Raf association by taking advantage of hsp70 release from K8/18 in the presence of Mg-ATP (Liao et al., 1995). Dissociation of hsp70 from K8/18 precipitates

Figure 1. Keratin association with Raf-1 kinase. (A) HT29 cells (lanes 1 and 2) were cultured in the presence or absence of OA (1 μg/ml, 2 h), then solubilized with 1% NP-40. Alternatively, transgenic mice (lanes 3–6) that overexpress human K18 (Ku et al., 2002) were injected with MLR (30 μg/kg) in saline (+) or with saline alone (−). After 2 h, the livers were homogenized with 1% NP-40. K8/18 immunoprecipitates were obtained from HT29 and liver NP-40 lysates then separated by SDS-PAGE. Duplicate gels were stained with Coomassie blue or transferred then blotted with Ab to Raf or 14-3-3. Note heat shock protein 70 (hsp70) association with K8/18. (B) BHK cells were transfected with vector alone, Raf, K8/18, K8/19, Raf+K8/18, or Raf+K8/19 constructs. After 3 d, transfected cells were solubilized followed by precipitation of K8/18 or K8/19 using K18- or K19-specific mAb. Immunoprecipitates (i.p.) were analyzed as in A. Arrow positioned between K18 and K19 highlights a nonspecific band, and arrowheads indicate previously characterized K18 fragments (Ku et al., 1997). (C) A total cell lysate and K8/18 precipitates were prepared from HT29 cells. After separation by SDS-PAGE, duplicate gels were stained with Coomassie blue (lanes 1 and 2) or transferred to a membrane for overlay assay (lanes 1 and 2'). The membrane was incubated with recombinant Raf kinase, washed, and blotted with anti-Raf Ab (lanes 1' and 2'). (D) K8/18 precipitates were prepared from HT29 cells then incubated (30 min, 22°C) in the absence or presence of Mg-ATP, followed by washing then blotting with Ab to Raf or hsp70. (E) HT29 cells were incubated in the presence or absence of H2O2 for 1 h followed by processing 80% of the cells for K8/18 precipitation and the remaining to prepare a total cell lysate. Precipitates were analyzed by blotting with the indicated Ab, including phospho-Erk1/2 as an indicator of Raf kinase activity.
leaves keratin-bound Raf intact (Fig. 1 D). Keratin–Raf dissociation also occurs upon exposure to oxidative stress, in association with: (a) increased K18 S33 phosphorylation; (b) keratin–14-3-3 binding; and (c) Raf kinase activation as determined by Erk1/2 phosphorylation (Fig. 1 E). Together, these results demonstrate keratin–Raf association, in a phosphorylation modulated and K8 dependent but hsp70 independent fashion, in cultured HT29 cells and mouse liver.

Given the keratin–Raf association, we asked if K8/18 is phosphorylated by Raf kinase. Recombinant human Raf-1 preferentially phosphorylated K18 (Fig. 2 A). Peptide mapping of the tryptic K18 phosphopeptides (Fig. 2 B), obtained after in vitro phosphorylation by Raf kinase, showed a nearly identical pattern to previously described physiologic K18 phosphorylation (Ku et al., 1998) at S33 (peptide 1) and S52 (peptide 2; Fig. 2 B). This was confirmed by in vitro phosphorylation of K8/18 immunoprecipitates obtained from BHK cells, cotransfected with wild-type (WT) K8 and WT or phosphorylation mutant K18. Mutation of K18 S52 abolishes in vitro phosphorylation of K18 by Raf kinase, whereas mutation of K18 S33 does not have a significant effect on overall K18 phosphorylation since its phosphorylation (i.e., Fig. 2 B, peptide 1) is relatively minor (Fig. 2 C). The K18 phosphorylation mutants were confirmed using pS52/pS33-specific Abs (Fig. 2 C). In vivo K18 phosphorylation by Raf was verified by comparing K18 S33 and S52 phosphorylation in BHK cells cotransfected with WT K8/18 and WT Raf kinase or the kinase-inactive Raf S259/S621A double mutant. Under these conditions, K18 S33/S52 phosphorylation increased in BHK cells that were transfected with WT Raf, but not Raf S259/S621A or vector (Fig. 2 D). This supports the in vitro kinase assay data and indicates that Raf is a likely physiologic K18 kinase that phosphorylates K18 primarily at S52/H11022 S33. Given the obligate and heteropolymeric noncovalent association of K8 and K18 in cells, the combined results of Figs. 1 and 2 suggest that K8 serves as an adaptor for Raf kinase, which in turn phosphorylates K18.

The observation that phosphatase inhibition dissociates keratin–Raf binding (Fig. 1 A), led us to test if keratin or Raf hyperphosphorylation, or both, cause release of the keratin–Raf complex. Reconstitution of in vitro–translated Raf kinase binding to K8/18 immunoprecipitates that were isolated from OA-treated or untreated HT29 cells was similar (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200402051/DC1). K18 S33/S52-to-Ala or K8 S23/S73/S431-to-Ala mutations (which abolish all known and major K8/18 phosphorylation sites) do not affect keratin–Raf binding as determined by cotransfection into BHK cells (Fig. S1 C). These keratin mutants behave similar to WT K8/18 in that they also release bound Raf in the cells treated with OA (not depicted). Hence, modulation of Raf, rather than K8, phosphorylation is likely to be responsible for dissociating the keratin–Raf complex.

The role of Raf phosphorylation was confirmed by cotransfection, into BHK cells, of K8/18 and a series of Ser-to-Ala single or double Raf mutants that inhibit known phosphorylation sites (S259/S621/S338/Y341/T491/S494).
Of these, two Raf constructs partially (S621A) or greatly (S259A/S621A) diminished keratin–Raf interaction, whereas other mutations had minimal effects (Fig. 3 A). Both Raf mutations resulted in deactivation of Raf kinase and hyper-phosphorylation of Raf S338 (Fig. 3 A, lanes 5 and 6), perhaps due to increased accessibility of Pak3 kinase to S338 after Raf S621A mutation (King et al., 1998). Raf kinase activity was determined by increased phospho-Erk1/2 as detected in cells transfected with WT Raf but not vector alone (Fig. 3 A, lanes 1 and 2). The Raf S338A mutation causes Raf S621 dephosphorylation and Raf kinase deactivation, but had a minimal effect on keratin–Raf association (Fig. 3 A, lane 7). In addition, a Raf R89L mutation that disrupts Ras–Raf binding (Fabian et al., 1994) did not affect keratin–Raf association. We also examined Raf phosphorylation and kinase activity in BHK cells under conditions of keratin–Raf complex formation or release (Fig. 3 B). Release of Raf is associated with its kinase activation (as determined by increased phospho-Erk1/2) and increased Raf S338/S621 phosphorylation. Together, the Raf pS259/pS621 sites that are involved in 14-3-3–Raf association (Tzivion et al., 1998) are also important in keratin–Raf association (S621>H259). Keratin–Raf association is independent of Ras–Raf interaction and does not require Raf kinase activity, whereas Raf dissociation from keratin occurs in concert with its activation. Quantitative immunoblotting indicated that ~2% of the total Raf pool binds to K8/18 (unpublished data). This fraction likely reflects mainly phosphorylated Raf.

Given that any two of the three K8/18, Raf, and 14-3-3 entities can associate with each other, we tested the dynamics of their association by in vitro reconstitution. K8/18 immunoprecipitates (prewashed with the detergent Empigen [Emp], which releases bound Raf and 14-3-3) were incubated with Raf or 14-3-3 proteins alone, together before or after premixing, or sequentially followed by washing then assessment of the keratin bound 14-3-3 or Raf. As shown in Fig. 4 A: (a) Raf and 14-3-3 binding to keratins can be reconstituted (lanes 3 and 4); (b) keratin-bound Raf blocks 14-3-3 binding but not vice versa (lanes 5 and 6); and (c) when Raf and 14-3-3 are added simultaneously, Raf bound to keratins preferentially though premixing yielded preferential Raf–14-3-3 binding (lanes 7 and 8). This indicates that Raf can bind to K8/18 efficiently, even in the presence of 14-3-3 proteins, and can also block keratin–14-3-3 binding.

We then tested the effect of mutating the Raf-binding motif of 14-3-3 on keratin–14-3-3 binding in vitro and in vivo. The 14-3-3ζ crystal structure suggests that positively charged residues in its amphipathic groove, including K49/ R56/R60, modulate diverse ligand binding (Liu et al., 1995; Xiao et al., 1995; Yaffe et al., 1997; Obsil et al., 2001). For example, 14-3-3ζ K49E or R56E decreased Raf–14-3-3 interaction, whereas R60E had subtle effects on 14-3-3–Raf binding (Zhang et al., 1997). As shown in Fig. 4 B, WT purified recombinant 14-3-3ζ but none of its mutants (K49E/ R56E/R60E) bind K8/18. Similar findings were obtained in BHK cells transfected with WT K8/18 and WT or mutant...
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flag-tagged 14-3-3ζ (Fig. 4 C). This indicates that the positively charged residues (K49/R56/R60) in the Raf-binding groove of 14-3-3ζ are critical for binding to K8/18.

The findings of this work indicate that keratin intermediate filaments can sequester a unique population of Raf-1 kinase and regulate its signaling potential in a Raf kinase phosphorylation-dependent fashion. This is similar to 14-3-3 regulation of Raf-1, and highlights the molecular domains and dynamic features of keratin–14-3-3–Raf interactions. For example, Raf S259/S621 regulates Raf interaction with 14-3-3 and K8/18 (Fig. 3), whereas 14-3-3 K49 and R56 regulate 14-3-3 interaction with Raf and K8/18 (Fig. 4). Given the overlap of 14-3-3 positively charged residues that regulate binding to keratins and to Raf kinase, our working model is phosphorylated Raf pS259/pS621, that makes up at least 2% of the total Raf pool, may interact with similar positively charged Arg/Lys residues on K8 that remain to be identified (Fig. 5 A). Some differences do distinguish keratin–14-3-3 versus Raf–14-3-3 association including the importance of 14-3-3 R60 for keratin–14-3-3

Figure 5. Keratin, Raf kinase, and 14-3-3 protein interactions. (A) Raf pS259/pS621 associates with positively charged residues (K49/R56/R60) on 14-3-3 and may interact with similar positively charged residues (K and R) on K8/18. (B) A schematic summarizing keratin–Raf association and release. K8/18 associates with Raf under basal conditions. Oxidative or toxin stress conditions cause dissociation of the keratin–Raf complex, and hyperphosphorylation of K8/18 (P) and Raf (pS) in association with Raf activation and K18 phosphorylation. Activation of Raf kinase also renders Raf available to phosphorylate other substrates.

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(this work) but not Raf–14-3-3 binding (Zhang et al., 1997). Keratin-bound Raf kinase is released from K8/18 during oxidative and some toxin-mediated stresses in association with its activation and phosphorylation of K18 (Fig. 1), thereby suggesting a novel mechanism for Raf-1 kinase activation (Fig. 5 B). Notably, keratin–Raf dissociation is independent of mitogenic stimuli including exposure to EGF or mitosis (not depicted), which is supported by Raf–keratin binding being unrelated to Ras–Raf association (Fig. 3 A).

Our results also provide strong evidence that K18 is a novel in vivo substrate for Raf-1 kinase (Fig. 2). Raf binding with K8/18 is independent of Raf’s kinase activity, which is consistent with its activation upon release from K8/18. Our working model is that inactive Raf binds with K8 then phosphorylates K18 upon its activation and release from K8 (Fig. 5). We cannot exclude the possibility that keratin phosphorylation by Raf-1 kinase may occur by the Raf pool that is not keratin bound.

The only other intermediate filament protein known to interact with 14-3-3 is vimentin, which binds to 14-3-3 after treatment with the phosphatase inhibitor calyculin A (Tzivion et al., 2000). Vimentin complexes indirectly with Raf kinase, via unknown components, and is phosphorylated by one or more kinase constituents of this complex but not by Raf itself (Janosch et al., 2000). The mechanism of regulation of the indirect Raf binding with vimentin has not been tested but may be different to that of keratin–Raf association, given the direct nature of the keratin–Raf association described herein and the finding that Raf S259/S621 phosphorylation is not essential for Raf–vimentin binding (Janosch et al., 2000). The direct K8/18–Raf association, independent of Raf–Ras binding and Raf kinase activity suggests a new role for keratins, which may extend to other intermediate filament proteins, in modulating some of Raf cell signaling events in epithelial cells.

Materials and methods

Cell lines and Abs

HT29 (human colon) and BHK-21 cells were cultured as recommended by the supplier (American Type Culture Collection). The Abs that were used (Ku et al., 1998) included: mAb L2A1, which recognizes human K18; rabbit Ab 8592, which recognizes hK18 pS33; and rabbit Ab 3055, which recognizes hK18 pS52. Additional Abs used were directed to: 14-3-3 (Promega); and recombinant 14-3-3 (Zhang et al., 1997). In vitro-translated [35S]-Raf was prepared by using the TNT coupled transcription/translation kit as recommended by the supplier. Whole cell extracts were prepared by solubilizing with Laemmlli sample buffer containing 2% SDS followed by blotting and visualization using ECL (Ku et al., 1998). The fraction of Raf that binds to K8 was estimated by comparison of an immunoprecipitated total lysates run on the same gel with Raf coimmunoprecipitated with K8/18. The overlay assay was performed as described previously (Liao et al., 1995) except for using in vitro-translated recombinant Raf kinase and Raf Ab.

Transgenic mice

For induction of keratin hyperphosphorylation, transgenic mice that over-express WT human K18 (Fu et al., 2000) were intraperitoneally injected with MLR in saline (30 µg/kg) or with saline alone. After 2 h, the livers were isolated then homogenized with 1% NP-40 in PBS followed by immunoprecipitation using mAb L2A1. The K8/18 precipitates were separated with SDS-PAGE then blotted with the indicated Ab.

Online supplemental materials

Fig. 5 demonstrates that keratin–Raf binding is independent of keratin phosphorylation. Online supplemental materials is available at http://www.jcb.org/cgi/content/full/jcb.200402051/DC1.

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