Heterogeneous Nuclear Ribonucleoprotein H Blocks MST2-Mediated Apoptosis in Cancer Cells by Regulating a-raf Transcription

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Abstract

A-Raf belongs to the family of oncogenic Raf kinases that are involved in mitogenic signaling by activating the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK pathway. Low kinase activity of A-Raf toward MEK suggested that A-Raf might have alternative functions. Here, we show that A-Raf prevents cancer cell apoptosis contingent on the expression of the heterogeneous nuclear ribonucleoprotein H (hnRNP H) splice factor, which is required for the correct transcription and expression of a-raf. Apoptosis was prevented by A-Raf through sequestration and inactivation of the proapoptotic MST2 kinase. Small interfering RNA–mediated knockdown of hnRNP H or A-Raf resulted in MST2-dependent apoptosis. In contrast, enforced expression of either hnRNP H or A-Raf partially counteracted apoptosis induced by etoposide. In vivo expression studies of colon specimens corroborated the overexpression of hnRNP H in malignant tissues and its correlation with A-Raf levels. Our findings define a novel mechanism that is usurped in tumor cells to escape naturally imposed apoptotic signals. Cancer Res; 70(4): 1679–88. ©2010 AACR.

Introduction

Heterogeneous nuclear ribonucleoprotein H (hnRNP H) is a member of the subfamily of hnRNPs, including hnRNP H, H′, F, and 2H9 (1). hnRNP H functions in the splicing of selected target mRNAs, such as c-src in neuronal cells (2), bcl-x (3, 4), plp/dm20 (5), Drosophila nanos (6), HIV-1 splicing substrates (7), and rodent tropomyosin (8). According to available knowledge of their functions, hnRNP F/H and hnRNP A/B are key players in alternative splicing (9). As shown more recently, they also have a role in generic splicing (i.e., in molecular mechanisms of intron definition; ref. 10). Overexpression of hnRNP H was described in various human cancers, including hepatocellular, pancreatic, and laryngeal carcinomas (11, 12). Whether and how hnRNP H is linked to the pathogenesis of cancer remains unexplored to date. We combined specific inhibition of hnRNP H expression with whole-genome transcriptome analysis, with the aim to analyze the phenotype of hnRNP H-deficient carcinoma cells. High levels of hnRNP H were mandatory for the expression of the full-length A-Raf protein, whereas it affected neither Raf-1 nor B-Raf protein expression. On knockdown of hnRNP H expression, a-raf mRNA levels were reduced. The expression of A-Raf was essential to sequester and deactivate the proapoptotic kinase MST2 and inhibit apoptosis. Thus, this study provides a rational explanation for the overexpression of hnRNP H in human tumors as a splicing regulator of the a-raf mRNA. In addition, it identifies a role for A-Raf as a potent inhibitor of the MST2 tumor suppressor pathway in carcinoma cells.

Materials and Methods

Immunohistochemistry. Carcinoma specimen and healthy tissue were obtained during routine biopsy or surgery after informed consent. Carcinoma and healthy tissue specimens were snap frozen, cryopreserved, and processed to generate serial sections (4 μm). For immunohistologic staining, polyclonal rabbit anti-human hnRNH H antibody (Bethyl Laboratories) and polyclonal goat anti-human A-Raf antibody (Santa Cruz Biotechnology) were used. Antigen-antibody complexes were visualized using the avidin-biotin-peroxidase method (13).
Cell lines. HeLa human cervical carcinoma cells, GHD-1 human hypopharynx carcinoma cells, HCT116 human colon carcinoma cells, and HEK293 human embryonic kidney cells were cultured in standard DMEM containing 10% FCS and passaged thrice each week. HeLa, HCT116, and HEK293 cell lines were purchased from either the American Type Culture Collection or Cancer Research UK. The cells were authenticated by the European Collection of Animal Cell Cultures in September 2009 using microsatellite genotyping (PCR based). GHD-1 is a self-established cell line from a hypopharynx head and neck squamous cell carcinoma (HNSSC) tumor (14).

Laser scanning fluorescence microscopy. Endogenous A-Raf and MST2 expression in HeLa carcinoma cells and human carcinoma specimen was analyzed using a fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope, Leica). For A-Raf, MST2, and cytochrome c detection, cells were fixed and stained with specific antibodies against A-Raf, MST2, and cytochrome c. Dye-coupled Alexa Fluor antibodies (Alexa Fluor 488 and Alexa Fluor 647, Molecular Probes) were used as secondary antibodies. Subsequently, Hoechst 33342 was used for labeling of nuclear DNA (Sigma). Confocal Software Lite (Leica) for evaluation and quantification was used according to the manufacturer's instructions.

Transfections. Transient transfections of small interfering RNAs (siRNA) and expression plasmids were conducted with MATra-A reagent (IBA) or the Nucleofactor system (Amaxa GmbH) according to the manufacturer's instructions. The total amounts of transfected DNA or siRNA were kept constant by filling up with empty vector or control siRNA as required. The following siRNA sequences were used: hnRNP H, 5′-GGAGCCUCGGCUUUGAGAGAdTdT-3′ (siRNA#1), 5′-GAAUAGGGCACAGGGUUAUdTdT-3′ (siRNA#2), and 5′-GCAAGGGAAGAUAUUUCAdTdT-3′ (siRNA#3; Eurogentec); A-Raf, Silencer Validated siRNA 151 (Ambion); MST2, 5′-GGAUAGUUUUUCAAUAGGdTdT-3′; and control siRNA, 5′-UCUGCGGUAUUUCUCAAUdTdT-3′.

Semiquantitative reverse transcription-PCR. Total RNA from cell lines was isolated using the High Pure RNA Isolation Kit (Macherey-Nagel), and cDNA was generated using the reverse transcription system (Promega) according to the manufacturer’s instructions. Total RNA from human tissues was isolated using the Precellys 24 Cell Lysis System (Bertin Technologies) and the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. cDNA was generated using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen) according to the manufacturer’s instructions.

We performed semiquantitative PCR for the expression of hnrnp H, Bcl-x, a-raf, glyceralddehde-3-phosphate dehydrogenase (GAPDH), and mapk7 (95°C for 30 s, annealing for 30 s, 72°C for 30 s). Amplicon size of the PCR is given in brackets: hnrnp H (385 bp), 5′-AAATGGGGCTCAGATTTGGC-3′ and 5′-GCTATCTCTGTAAGGAAACGTCG-3′; A-Raf (210 bp), 5′-ATGAGGCCACCCAGGGGCGC-3′ and 5′-CGTCTTCTCCCTTGTGAGTC-3′; Bcl-x (x1 isofrom, 456 bp; x2 isofrom, 267 bp), 5′-ATGCGACGATTAAGGCAAGCG-3′ and 5′-TCATTTCGACTGAGAGTG-3′; GAPDH (258 bp), 5′-GTGCCTCTTGAAGTGACAGAGGAG-3′ and 5′-AGAACATCATCCCTGCTCTTACTG-3′; and MAPK1 (233 bp), 5′-CTTCCAACTCGTCGTCACAC-3′ and 5′-GGAAAAGATGGCCTGTGATAGAACG-3′. Image software (NIH, Bethesda, MD) was used for quantification of RT-PCRs.

Immunoprecipitations. Hemagglutinin (HA)–A-Raf was immunoprecipitated using immobilized monoclonal mouse anti-HA tag antibody 3F10 (Roche Diagnostics). Flag-MST2, Flag-A-Raf, and Flag–B-Raf were immunoprecipitated using immobilized monoclonal mouse anti-Flag antibody M2 (Sigma). Endogenous A-Raf was immunoprecipitated using the polyclonal goat anti-human A-Raf antibody. Endogenous MST2 was immunoprecipitated using the polyclonal goat anti-human MST2 antibody (Santa Cruz Biotechnology). As an isotype control, the polyclonal goat anti-human enolase antibody (Santa Cruz Biotechnology) was used. HA-tagged A-Raf was immunoprecipitated using the monoclonal mouse anti-HA tag antibody 3F10. All antibodies were covalently bound to protein G-Sepharose (Amerham) as described elsewhere (15) to avoid interference of immunoglobulin heavy chain bands with the similar-sized MST2 protein. Cells were lysed in 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.5% NP40 supplemented with protease and phosphatase inhibitors (Roche Diagnostics). Lysates were cleared of debris by centrifugation (20,000 × g, 10 min) and the supernatant was used for immunoprecipitation. The immunoprecipitates were washed thrice with lysis buffer and separated by SDS-PAGE and immunoblotted.

Immunoblot analysis. Protein lysates or immunoprecipitates were resolved by SDS PAGE (10–15%) and blotted on polyvinylidene difluoride membrane (Millipore). Protein visualization was performed using the following antibodies in combination with horseradish peroxidase–conjugated secondary antibodies and the enhanced chemiluminescence system (GE Healthcare): polyclonal rabbit anti-human hnRNPH antibody, polyclonal goat anti-human A-Raf antibody, polyclonal rabbit anti-human MST2 antibody (Stratagene), polyclonal rabbit anti-human Bcl-xL antibody (Cell Signaling), monoclonal mouse anti-human actin antibody (Santa Cruz Biotechnology), monoclonal mouse anti-human tubulin antibody (Santa Cruz Biotechnology), monoclonal mouse anti-human c-Myc antibody (Santa Cruz Biotechnology), monoclonal mouse anti-human Flag antibody (Sigma), monoclonal mouse anti-human c-Raf-1 antibody (Becton Dickinson), polyclonal rabbit anti-human B-Raf antibody (Santa Cruz Biotechnology), monoclonal rabbit anti-human MST2 (N terminus) antibody (Epitomics, Inc.), polyclonal rabbit anti-human mitogen-activated protein (MAP) kinase [extracellular signal-regulated kinase (ERK) 1 and ERK2] antibody (Sigma), monoclonal mouse anti-human MAP kinase activated (diphosphorylated ERK1 and ERK2) antibody (Sigma), polyclonal rabbit anti-human Puma antibody (Sigma), polyclonal rabbit anti-human caspase-3 antibody (Santa Cruz Biotechnology), monoclonal rabbit anti-human caspase-3

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antibody (8G10; Santa Cruz Biotechnology), and monoclonal mouse anti-human poly(ADP-ribose) polymerase (PARP) antibody (Becton Dickinson).

**MST2 kinase activity assay.** MST2 immunoprecipitates or cell lysates were separated by SDS-PAGE using gels that were copolymerized with 0.5 mg/mL myelin basic protein. In-gel kinase assays were performed as described previously (16), except that after the final wash gels were soaked in 50 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl2, and 2 mmol/L DTT for 30 min before the addition of 50 μmol/L ATP, including 1.85 MBq [γ-32P]ATP, in a total volume of 10 mL.

**Apoptosis assays.** Attached and floating cells were harvested, washed with PBS, and fixed in 80% ethanol. Subsequently, cells were incubated with RNase A (10 units; Roche) and stained with propidium iodide (50 μg/mL) before analysis on a fluorescence-activated cell sorting flow cytometer (BD Clontech). Apoptosis was determined by measuring subgenomic DNA content.

**Image analysis and quantification.** Relative expression levels from RT-PCRs and immunoblots were assessed using Quantity One software according to the manufacturer’s instructions (Bio-Rad).

**Statistical analysis.** Significance levels were determined by two-tailed Student’s t test analyses using Microsoft Excel. Due to the non-normal distribution of the expression analysis data (RT-PCR), results are given as the median with the interquartile range (IQR). For comparison of hnRNP H and A-Raf expression between sample groups, we used the Mann-Whitney U test. The association between hnRNP H and expression of A-Raf was tested using Pearson correlation. All tests were two-sided and results were considered significant if \( P < 0.01 \). Calculations were carried out with Minitab 15 software (Minitab Ltd.).

**Results**

**hnRNP H is overexpressed in carcinomas.** hnRNP H was overexpressed in head and neck carcinomas, whereas normal mucosa samples displayed low levels of hnRNP H, with the exception of an intermediate expression in cells of the basal membrane layer (Fig. 1). hnRNP H was overexpressed in the vast majority of head and neck carcinoma (i.e., in 60% of oropharynx, 80% of hypopharynx, 100% of larynx carcinomas, and 67% of lymph node metastases; Supplementary Table S1). Dysplasia of the head and neck area also showed an increase in hnRNP H expression (data not shown). In normal tissue, hnRNP H was absent or only faintly detectable in most human tissues, including muscle, heart, liver, kidney, and pancreas samples. Prostate, gastric, and gut epithelium as well as spleen and testis cells showed an intermediate expression of hnRNP H as compared with carcinoma samples (Supplementary Fig. S1).

**Downregulation of hnRNP H induces apoptosis.** Knockdown of hnrnp H in HeLa (cervix), GHD-1 (hypopharynx), and HCT116 (colon) cells via RNA interference (Fig. 2A; Supplementary Fig. S2A and B) reduced cell numbers (Fig. 2B) by induction of apoptosis, as evidenced by activation of caspase-3, PARP cleavage, and 10-fold increases in DNA fragmentation with up to 50% of cells displaying a pronounced sub-G1 DNA content (Fig. 2A). Decreased cell numbers could be completely rescued by exogenous hnRNP H expression (Supplementary Fig. S2C) and were hence not the result of off-target effects of the siRNAs used. Transient hnRNP H overexpression also protected against etoposide-induced apoptosis by 50% (Fig. 2C), confirming that hnRNP H has antiapoptotic properties both under normal growth conditions and under cytotoxic drug challenge.

**A-Raf and Bcl-xl are splicing targets of hnRNP H.** As hnRNP H is a splicing factor, we performed a genome-wide cDNA microarray screening to profile hnRNP H target mRNAs potentially involved in apoptosis (data not shown). Downregulation of hnRNP H caused a decrease in antiapoptotic Bcl-x\(_l\) and A-Raf mRNA and protein levels (Fig. 3A and B), which theoretically could promote apoptosis. hnRNP H specifically regulated the expression of wild-type A-Raf, leaving Raf-1 or B-Raf unaffected (Fig. 3B). Transfection of A-Raf, but not Bcl-x\(_l\), prevented apoptosis resulting from hnRNP H repression (Fig. 3C). Double staining of hnRNP H and A-Raf

![Figure 1](https://www.aacrjournals.org/cancerres/70(4)/1681/figure1.png)
in HNSCC sections and single stainings of consecutive tumor sections revealed a tight correlation between A-Raf and hnRNP H expression at the single-cell level (Fig. 3D; Supplementary Fig. S3A–F).

A-Raf counteracts apoptosis by controlling MST2 activity. To test whether A-Raf mediates hnRNP H apoptosis protection, its expression was suppressed by siRNA (Fig. 4A and B). Knockdown of a-raf caused a 3-fold reduction in cell numbers (Fig. 4A) and an increase in apoptotic cells similar to the levels observed after hnRNP H inhibition (Fig. 4B). Likewise, A-Raf overexpression counteracted etoposide-mediated apoptosis comparably with hnRNP H (Fig. 4C).

A role in tumor suppression was suggested for the mammalian MST1/2 kinases based on their ability to induce cancer cell apoptosis (17–20). Raf-1 suppressed MST2-mediated apoptosis by binding to and inhibiting the enzymatic activity of MST2, whereas B-Raf barely interacted with MST2 (16). Transfection of A-Raf inhibited MST2 kinase activity in a dose-dependent fashion (Fig. 5A). In contrast, the knockdown of either hnrnp h or a-raf augmented MST2 kinase activity, which was fully
prevented by complementation with an A-Raf expression plasmid when knocking down hnRNP H (Fig. 5B). MST kinases are cleaved by caspases, releasing a 36-kDa kinase moiety that can translocate to the cell nucleus where it phosphorylates substrates involved in apoptotic processes (17, 21–25). MST2 cleavage and activation of caspase-3 occurred after knockdown of hnRNP H (Supplementary Fig. S4A and B), further confirming that the reduction of hnRNP H expression leads to a critical deregulation of MST2. Additionally, knockdown of either hnRNP H or A-Raf resulted in the activation of caspase-3 and PARP and an upregulation of the proapoptotic protein Puma (Supplementary Fig. S4A), indicating that downstream targets of the MST2 pathway are activated (26). Inhibition of MST2 expression with siRNA fully abrogated apoptosis induced by hnRNP h or araf knockdown, confirming that hnRNP H and A-Raf regulation of apoptosis was MST2 dependent (Fig. 5C).

**A-Raf and MST2 interact.** Raf-1–mediated inhibition of MST2 effects was dependent on an interaction of both proteins (16). Transfected HA–A-Raf and Flag-MST2 as well as endogenous A-Raf and MST2 interacted (Fig. 5D).

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**Figure 3.** hnRNP H controls bcl-x and a-raf mRNA levels and splicing, but only A-Raf antagonizes apoptosis induced by hnRNP H depletion. A, bcl-x, a-raf, and hnRNP H mRNA expression in HeLa cells was analyzed by semiquantitative RT-PCR 4 d after transfection with control (Ctrl. siRNA) or hnRNP H–specific siRNA (siRNA#1). gapdh mRNA levels were determined as loading controls. Shown is a representative result from three independent experiments. B, Bcl-xl, A-Raf, Raf-1, B-Raf, and hnRNP H protein expression was analyzed by immunoblotting 3 d after transfection with control (Ctrl. siRNA) or hnRNP H–specific siRNA (siRNA#1). As loading control, tubulin protein levels were determined. Shown are representative results from three independent experiments. C, HeLa cells were cotransfected with A-Raf or Bcl-xl expression plasmids and siRNAs as indicated. Apoptosis was determined assessing DNA fragmentation by flow cytometry 3 d after transfection. Top, columns, mean percentage of apoptosis of three independent experiments; bars, SD. Bottom, hnRNP H, A-Raf, and Bcl-xl expression was assessed by immunoblotting. D, hnRNP H and A-Raf colocalize in primary carcinomas. hnRNP H and A-Raf were visualized using double staining with the respective antibodies.
In addition, A-Raf–K336M, a catalytically inactive mutant, A-Raf–Y301D, a kinase-active mutant, and A-Raf–YY301/302FF, a nonactivatable mutant, interacted with MST2 similarly as A-Raf (Supplementary Fig. S5A), hence indicating that kinase activity is dispensable for the inhibitory effects of A-Raf on MST2. In contrast to the interaction of Raf-1 with MST2, which is disrupted by growth factors (Supplementary Fig. S5B; ref. 16), interaction between A-Raf and MST2 revealed largely resistant (Supplementary Fig. S5B). As shown before (16), an interaction of the third isoform, B-Raf, with endogenous MST2 is hardly detectable (Supplementary Fig. S5B) and may occur via heterodimerization of B-Raf and Raf-1 (16).

Endogenous localization of A-Raf and MST2 was visualized in HeLa cells (Supplementary Fig. S6A). Both proteins appeared as cytoplasmic speckles, which perfectly merged in digital overlays (Supplementary Fig. S6B). These speckles represented mitochondria as shown by costaining with cytochrome c, indicating that A-Raf–MST2 interaction occurs at mitochondria. Importantly, the results obtained in cultured cancer cells were corroborated in human HNSCC (Supplementary Fig. S6B; ref. 16), and neck carcinomas, hnRNP H and A-Raf were both overexpressed in tumors. Next, endogenous mRNA expression levels of hnrnp h and a-raf were analyzed in a series (n = 29) of human Dukes B colon carcinomas and autologous adjacent nonmalignant tissues by semiquantitative RT-PCR (Fig. 6A). As a basic principle, hnrnp h and a-raf mRNA levels correlated, independently of the tissue analyzed. Expression levels of hnrnp h and a-raf in normal tissue were then set to one for a comparison with the autologous carcinomas. A median relative expression of 2.6-fold for hnrnp h and 1.7-fold for a-raf in tumor specimens was calculated, indicating that hnrnp h and a-raf are overexpressed in carcinomas. hnrnp h overexpression significantly correlated with increased levels of a-raf at the single patient level (r² = 0.564; P < 0.001; Fig. 6B). When patients were divided into three groups according to their relative expression levels of hnRNP H and a-raf mRNA in tumor tissue (Fig. 6B), a significantly elevated number of patients showed both high expression of hnRNP H (n = 16/29; χ² = 21.8; P = 0.001) and a-raf (n = 11/29; χ² = 16.7; P = 0.001; Fig. 6B). Comparing the relative expression of hnRNP H and a-raf mRNA in normal and tumor tissues (Fig. 6C), we found that in tumor tissues the expression of hnRNP H (406; IQR, 278–462) was significantly higher than in normal tissues (137; IQR, 100–290; P = 0.001), corresponding to a 3-fold increase. Expression of a-raf was overexpressed in carcinomas. As suggested from immunohistochemistry with head and neck carcinomas, hnRNP H and A-Raf were both overexpressed in tumors. Next, endogenous mRNA expression levels of hnrnp h and a-raf were analyzed in a series (n = 29) of human Dukes B colon carcinomas and autologous adjacent nonmalignant tissues by semiquantitative RT-PCR (Fig. 6A). As a basic principle, hnrnp h and a-raf mRNA levels correlated, independently of the tissue analyzed. Expression levels of hnrnp h and a-raf in normal tissue were then set to one for a comparison with the autologous carcinomas. A median relative expression of 2.6-fold for hnrnp h and 1.7-fold for a-raf in tumor specimens was calculated, indicating that hnrnp h and a-raf are overexpressed in carcinomas. hnrnp h overexpression significantly correlated with increased levels of a-raf at the single patient level (r² = 0.564; P < 0.001; Fig. 6B). When patients were divided into three groups according to their relative expression levels of hnRNP H and a-raf mRNA in tumor tissue (Fig. 6B), a significantly elevated number of patients showed both high expression of hnRNP H (n = 16/29; χ² = 21.8; P = 0.001) and a-raf (n = 11/29; χ² = 16.7; P = 0.001; Fig. 6B). Comparing the relative expression of hnRNP H and a-raf mRNA in normal and tumor tissues (Fig. 6C), we found that in tumor tissues the expression of hnRNP H (406; IQR, 278–462) was significantly higher than in normal tissues (137; IQR, 100–290; P = 0.0001), corresponding to a 3-fold increase. Expression of a-raf
(328; IQR, 174–404) was also significantly higher than in normal tissue (186; IQR, 138–276; \( P = 0.0026 \)), corresponding to a 1.8-fold increase.

**Discussion**

Tissue homeostasis and tumor suppression are in part maintained by naturally imposed proapoptotic events. Rapidly proliferating cells, including tumor cells, depend on antiapoptotic signals that counteract intrinsic and extracellular death-promoting factors to survive (27, 28). Various carcinoma entities present an overexpression of the nuclear factor hnRNP H (11, 12). Here, we show that hnRNP H promotes the proper generation of \( a-raf \) mRNA to express full-length A-Raf protein. As a member of the Raf family, A-Raf was long seen as a kinase, although with poor enzymatic potential. As a result of hnRNP H inhibition, an additional variant of \( a-raf \) mRNA was detectable, which probably represented a shortened, alternatively spliced variant. Whether this shortened mRNA gives rise to a variant

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**Figure 5.** hnRNP H and A-Raf suppress apoptosis via inhibition of MST2 kinase activation. A, increasing amounts of Flag–A-Raf expression plasmids were transiently transfected into HEK293 cells, and MST2 immunoprecipitates were examined for kinase activity using an in-gel kinase assay. An equal aliquot was used for immunoblotting to assure that equal amounts of MST2 had been immunoprecipitated. Lysates were immunoblotted for expression of the transfected Flag–A-Raf and tubulin as loading control. Shown are the representative results from three independent experiments. B, HeLa cells were transiently transfected with hnRNP H (siRNA#1), A-Raf, and control siRNAs at final concentrations of 100 nmol/L. Where indicated, A-Raf expression plasmid (1.0 \( \mu \)g) was cotransfected. Top, the kinase activity of MST2 immunoprecipitates was assessed by in-gel kinase assays at days 1 and 2 after transfection. Bottom, the amount of MST2 immunoprecipitated from whole-cell lysates was visualized by immunoblotting. C, HeLa cells were transfected with hnRNP H siRNA, A-Raf siRNA, or MST2 siRNA as indicated. Total amounts of siRNA were adjusted using control siRNA. Three days after transfection, apoptosis was determined by assessing DNA fragmentation using flow cytometry. Top, columns, mean of three independent experiments; bars, SD. Bottom, in parallel, the expression of hnRNP H, A-Raf, and MST2 was monitored in whole-cell lysates by immunoblotting. D, A-Raf and MST2 interact in cultured cells. Left, Flag-tagged MST2 and HA-tagged A-Raf were transiently transfected in HEK293 cells. Top, after 24 h, MST2 and A-Raf were immunoprecipitated (IP) with Flag tag–specific or HA tag–specific antibodies and analyzed by immunoblotting (WB). Bottom, right, lysates were immunoblotted for expression of the transfected HA–A-Raf, Flag-tagged MST2, and tubulin as loading control. Endogenous A-Raf and MST2 were immunoprecipitated from lysates of HeLa cells, which had been serum starved (0.1% FCS, 16 h) or treated full medium (10% FCS) after serum starvation using specific antibodies for A-Raf or MST2, and analyzed by immunoblotting. Top, as an isotype control, an antibody specific for enolase was used. Bottom, lysates were immunoblotted for expression of A-Raf, MST2, phosphorylated ERK1/2 (Phospho-ERK1/2), ERK1/2, and tubulin as loading control. HC, heavy chain immunoglobulin. Shown are representative results from three independent experiments.
A-Raf protein and what functions are assigned to this potential protein remains to be elucidated.

Knowledge of a pathophysiologic role of A-Raf in cancer cells is rather scarce when compared with Raf-1 and B-Raf. Especially the comparatively low capacity of A-Raf to phosphorylate MAP/ERK kinase (MEK), the only widely accepted substrate for Raf kinases was suggestive of alternative functions of A-Raf (29, 30). Our results suggest that a physiologic relevance of maintaining high-level expression of A-Raf is to prevent apoptosis by antagonizing the proapoptotic kinase MST2. This pathway has emerged as an important mode of control of apoptosis both in Drosophila melanogaster, where the MST2 orthologue is called Hippo (21), and in mammals (17). Downstream MST2 substrates such as Lats and hWW45 (called Warts and Salvador in Drosophila melanogaster, respectively) have been identified as tumor suppressor genes in mammals (31). Raf-1 has been shown to interfere with MST2 dimerization and phosphorylation by directly binding MST2 (16). As a result, MST2-mediated apoptosis was prevented. The B-Raf isoform did not bind MST2 and could not compensate for Raf-1 in this respect (16). Our results show that the third Raf family member, the A-Raf isoform (32), can efficiently repress MST2 activity and induction of apoptosis. Interestingly, this capability depends on the presence of hnRNP H, which enhances the generation of full-length A-Raf. MST2 and A-Raf were concomitantly overexpressed and colocalized at mitochondria in cancer cell lines and also in primary human tumors. The significance of this colocalization at mitochondria warrants further investigation but may explain why in human cancers A-Raf seems more efficient in inhibiting MST2 proapoptotic activity than Raf-1. A role for the potential shortened variant of A-Raf in binding MST2 is as yet unknown. A short isoform of A-Raf might lack the
ability to bind to MST2 and thus might be ineffective in repressing apoptosis.

In mice, a-raf knockout resulted in a severe phenotype in X−/Y males and in X+/X− females, hence in homozygous knockouts as a-raf maps to the X chromosome. Deficient animals died within 7 to 21 days after birth, displaying abnormalities in colon organogenesis and neurologic defects resulting in abnormal movement and proprioception. Although in vivo biochemical evidence is missing, these pathologic phenotypes seem reconcilable with increased apoptosis due to a lack of A-Raf control over the MST2 pathway (33, 34). Inhibition of MST2 requires neither the kinase activity of Raf-1 (16) nor the kinase activity of A-Raf. Such an antia apoptotic, kinase-independent function would be consistent with a comparably low MEK kinase activity of A-Raf (35) and was corroborated by the interaction of MST2 with a kinase-deficient variant of A-Raf. However, A-Raf still participates in ERK pathway as shown by double knockouts of Raf-1 and A-Raf in mouse embryos (36), although an inverse correlation between the kinase activity of Raf homologues and the capacity to interact with MST2 emerges from the data available (16).

In summary, our study presents three central findings: (a) A-Raf is a potent inhibitor of MST2-dependent apoptosis, (b) hnRNP H is necessary for proper splicing of mature A-Raf, and (c) the hnRNP H−A-Raf−MST2 axis seems instrumental in human tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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