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Research Article

Role of the Tumor Suppressor RASSF1A in Mst1-Mediated Apoptosis

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Abstract

Mammalian sterile 20–like kinase 1 (Mst1) is activated by both caspase-mediated cleavage and phosphorylation in response to apoptotic stimuli, including Fas ligation. Here, we examined the possible role of the tumor suppressor RASSF1A in Mst1 activation and Mst1-mediated apoptosis induced by death receptor signaling. Immunoprecipitation and immunofluorescence analyses revealed that Mst1 was associated with RASSF1A in cultured mammalian cells, with both proteins colocalizing to microtubules throughout the cell cycle. Whereas purified recombinant RASSF1A inhibited the kinase activity of purified recombinant Mst1 *in vitro*, overexpression of RASSF1A increased the kinase activity of Mst1 in intact cells, suggesting that regulation of Mst1 by RASSF1A *in vivo* involves more than the simple association of the two proteins. Both the activation of Mst1 and the incidence of apoptosis induced by Fas ligation were markedly reduced in cells depleted of RASSF1A by RNA interference and were increased by restoration of RASSF1A expression in RASSF1A-deficient cells. Moreover, the stimulatory effect of RASSF1A overexpression on Fas-induced apoptosis was inhibited by depletion of Mst1. These findings indicate that RASSF1A facilitates Mst1 activation and thereby promotes apoptosis induced by death receptor signaling. (Cancer Res 2006; 66(5): 2562-9)

Introduction

Mammalian sterile 20–like kinase 1 (Mst1) is a member of a family of serine/threonine kinases that show similarity to Ste20, an upstream activator of the mitogen-activated protein kinase pathway in budding yeast (1, 2). The Ste20-related catalytic domain of Mst1 is released from inhibition by a negative regulatory domain as a result of caspase-mediated cleavage. Such cleavage is triggered either by activation of death receptors, such as Fas and the tumor necrosis factor-α receptor, or by exposure of cells to inducers of apoptosis, such as staurosporine or ceramide (3–6). Whereas intact Mst1 is localized predominantly to the cytoplasm, the catalytic fragment of Mst1 generated by caspase-mediated cleavage translocates to the nucleus and phosphorylates histone H2B on Ser^{14}, resulting in chromatin condensation, DNA fragmentation, and, ultimately, cell death by apoptosis (7, 8). In addition to its activation by proteolytic cleavage, treatment of cells with okadaic acid, a phosphatase inhibitor, induces Mst1 activation, suggesting that the phosphorylation state of Mst1 affects its kinase activity (5, 9, 10). Several phosphorylation sites of human Mst1 have been identified, including Thr^{183}, one of the sites for Mst1 activation (10).

Recent studies have implicated Mst1 and the related protein Mst2 in regulation of cell cycle progression as well as in tumor suppression. A *Drosophila* homologue of Mst1/2, Hippo (Hpo), together with Salvador (Sav) and Warts (Wts), promotes both proper exit from the cell cycle and apoptosis during development (11–13). Hpo phosphorylates and activates the kinase Wts, and this process is facilitated by the scaffolding protein Sav (13). Such Hpo-Sav-Wts signaling results in cell cycle arrest and apoptosis both through suppression of transcription of the *cyclin E* (11–13) and *Drosophila* apoptosis inhibitor *diap1* (13) genes and through phosphorylation and consequent degradation of DIAP1 (11, 12).

Both Sav and Wts have human homologues, WW45 and Lats1/2, respectively, which are thought to be tumor suppressors (14, 15), and the Hpo-Sav-Wts signaling pathway seems to be conserved in mammalian cells (14–16). Mst1 and Mst2 have also been shown to associate with members of the RASSF family of tumor suppressors, all of which contain a conserved Ras-association (RA) domain (17, 18). RASSF proteins have been suggested to participate in the Hpo-Sav-Wts pathway in mammalian cells in a manner dependent on a protein-protein interaction domain, SARAH, that is shared by Sav, RASSF, and Hpo (19, 20). The *RASSF1* gene is located in a region of human chromosome 3p21.3 that frequently manifests allelic loss in many types of solid tumor (21). Two major products of *RASSF1*, RASSF1A and RASSF1C, both contain the Ras-association domain followed by the SARAH domain in their COOH-terminal regions but differ in their NH_2-terminal sequences (21). RASSF1A, but not RASSF1C, has been shown to function as a tumor suppressor. *RASSF1A* is thus frequently inactivated in lung, breast, and other cancer cells as a result of hypermethylation of a CpG island in its promoter (21–24), and restoration of RASSF1A expression was shown to suppress tumor cell growth *in vivo* and *in vitro* (21–23). Moreover, deletion of *RASSF1A* in mice that still expressed intact RASSF1C increased the frequency of spontaneous tumor development (25, 26).

RASSF1A is thought to function in the regulation of both the cell cycle and apoptosis. It inhibits cell cycle progression at the G_1-S transition by preventing the accumulation of cyclin D1 (27) and by interacting with the transcription factor $p120^{E4F}$ (28). Mitotic progression is also regulated by RASSF1A as a result of its ability to inhibit the activity of the APC-Cdc20 ubiquitin ligase complex and thereby to prevent the premature degradation of cyclins A and B (29, 30). Moreover, the association of RASSF1A with microtubules has been proposed to contribute to its function in cell cycle regulation as well as to modulation of microtubule dynamics (31, 32). In addition, RASSF1A has been implicated in Ras-mediated apoptotic signaling through its heterodimerization with novel Ras effector 1 (NORE1; ref. 33) as well as through its binding to Mst1 (17, 18), likely together with the Raf-1-binding protein CNK1 (17, 34). Intriguingly, it has been shown that coexpression of

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RASSF1 or NORE1 suppresses Mst1 kinase activity to undergo autoactivation *in vitro* and *in vivo*, but Ras(G12V) bound via NORE1A to Mst1 partially overcomes the inhibition of Mst1 autoactivation caused by NORE1A, suggesting that RASSF1 or NORE1 may restrict Mst1 activation (18). However, it still remains to be determined how the Mst-RASSF1 complex contributes to the regulation of physiologic apoptosis such as that induced by Fas activation in addition to Ras-regulated apoptosis (35). Here, we show that the tumor suppressor RASSF1A is required for full activation of Mst1 during Fas-induced apoptosis and, as a result, enhances Mst1-mediated apoptosis *in vivo*.

Materials and Methods

Plasmid construction. Expression vectors (pME18S) for Flag epitope–tagged forms of human Mst1, Mst1-N (residues 1-326), Mst1-C (residues 327-487), Mst1(K59R), Mst2, and Mst2(K56R) have been described previously (4). The cDNAs for Mst1(1-394) and Mst1(1-430) were amplified from the pME18S-Flag-Mst1 vector by the PCR and inserted into pME18S-Flag. The cDNAs for human RASSF1A-N (residues 1-119), RASSF1A-C (residues 120-340), and RASSF1A-Ras-association (residues 1-288) were amplified by the PCR and cloned into pCMV-HA, which was modified from pcDNA3 (Invitrogen, Carlsbad, CA), for expression of hemagglutinin (HA) epitope–tagged proteins. The cDNA for the RASSF1A(L301P) mutant was generated by site-directed mutagenesis. A vector for human RASSF1A mRNA-specific small interfering RNA (siRNA; ref. 29) or green fluorescent protein (GFP) siRNA (36) have been described previously, and a vector for Mst1 siRNA was generated by cloning annealed oligonucleotides derived from human Mst1 cDNA (sense, 5′-TGTGAAACTGAAACGCCAG-3′) into pSUPER (Oligoengine, Seattle, WA).

Cell culture and transfection. 293T, HeLa, NCI-H1299, and U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). A549 cells were cultured in RPMI 1640 supplemented with 10% FBS. Transient transfection of 293T cells or other cell types was done with the use of calcium precipitation or the Effectene reagent (Qiagen, Valencia, CA), respectively. For stable expression of GFP siRNA or RASSF1A siRNA in HeLa cells as well as Mst1 siRNA in U2OS cells, the cells were cotransfected with the corresponding pSUPER vector and pcDNA-puro. A549 cell line or NCI-H1299 cell line stably expressing RASSF1A were generated by retroviral infection as previously described (29). All stable cell lines were selected and cultured in the presence of puromycin (3 μg/mL).

Antibodies. A mouse monoclonal antibody to Mst1 (G2B) was used for immunoprecipitation as previously described (4), rabbit polyclonal antibodies to Mst1 (Cell Signaling Technology, Beverly, MA) were used for immunoblot analysis, and guinea pig polyclonal antibodies to Mst1, which were generated by injection of animals with purified recombinant hexahistidine (His_6)-tagged Mst1(K59R), were used for immunostaining. Mouse polyclonal antibodies to RASSF1A (A4) have been described previously (29), and antibodies to RASSF1A used for immunostaining were produced by injection of rabbits with purified His_6-tagged recombinant human RASSF1A. Other antibodies included mouse monoclonal antibodies to Flag (Clone M2; Sigma, St. Louis, MO), HA (Roche, Indianapolis, IN), β-actin (Sigma), and glutathione *S*-transferase (GST; Santa Cruz Biotechnology, Santa Cruz, CA) as well as rabbit polyclonal antibodies to histone H2B phosphorylated on Ser^{14} (Upstate Biotechnology, Waltham, MA), histone H2B (Upstate Biotechnology), and His_6 tag (Santa Cruz Biotechnology).

Purification of proteins expressed with a baculovirus system. The cDNAs for human Mst1 and Mst2 were cloned into pVL1393 (BD Biosciences, San Jose, CA) for expression of proteins with a COOH-terminal His_6 tag, and were subsequently introduced into Hi5 cells with a baculovirus expression system (BD Biosciences). The cells were harvested 3 days after infection, resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, and 0.1% Triton X-100], and subjected to ultrasonic treatment. The recombinant proteins were purified by Ni-NTA ion-exchange (HiTrapQ; Amersham Biosciences, Chalfont St. Giles, United Kingdom) and size-exclusion (Superdex200, Amersham Biosciences) chromatography.

Immunofluorescence analysis. HeLa cells plated on coverslips in 12-well dishes were washed with PBS, fixed in ice-cold methanol for 20 minutes, and incubated for 30 minutes at room temperature in PBS containing 1% bovine serum albumin. They were subsequently incubated overnight at 4°C with primary antibodies in the latter solution, washed thrice with TBS, and incubated with Cy3-conjugated goat antibodies to guinea pig IgG (The Jackson Laboratory, West Grove, PA) and Alexa Fluor 546–conjugated goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). The coverslips were mounted on glass slides in Vectorshield medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images acquired with an Olympus microscope equipped with a Hamamatsu Orca charge-coupled device camera were processed with Adobe Photoshop 8.0 software.

Assay of Mst activity. Cells were lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5% Triton X-100, phosphatase inhibitor mixture (1 mmol/L NaF, 1 mmol/L Na_3VO_4, and 1 mmol/L β-glycerol phosphate), and protease inhibitor mixture (1 mmol/L phenylmethylsulfonylfluoride, 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A). Immunoprecipitation of Mst1 from cell lysates was done as previously described (4), and the precipitates were washed extensively with lysis buffer and then with PBS containing 0.5 mol/L LiCl. The washed precipitates were incubated for 20 minutes at 30°C with 1.5 μg histone H2B (Roche) in 25 μL kinase assay buffer comprising 40 mmol/L HEPES-NaOH (pH 7.4), 20 mmol/L $MgCl_2$, 1 mmol/L DTT, phosphatase inhibitor mixture, 10 μmol/L unlabeled ATP, and 1 μCi [γ-^{32}P]ATP. Alternatively, the kinase activity of purified baculovirus-expressed His_6-Mst1 or His_6-Mst2 was assayed in the absence or presence of a purified bacterially expressed fusion protein of GST-RASSF1A. The reaction was terminated by the addition of Laemmli sample buffer and phosphorylated proteins were detected by SDS-PAGE followed by autoradiography.

Detection of apoptotic cells. Cells were grown on chamber slides (LabTakII; Nunc, Naperville, IL), treated with antibodies to Fas (CH-11; Upstate Biotechnology) and cycloheximide (Sigma), as indicated, and then stained with DAPI and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reagents (Roche). The proportion of apoptotic cells was determined as the percentage of TUNEL-positive cells among all DAPI-stained cells. At least 1,000 cells were counted for each sample, and three independent experiments were done.

Results

RASSF1A interacts with Mst1 *in vivo*. Because the members of RASSF family protein have been shown to interact with Mst1 kinase (17, 18), we first confirmed that RASSF1A associates with Mst1 *in vivo* by immunoprecipitation analysis. HA-tagged RASSF1A coprecipitated with Flag-tagged Mst1 and vice versa from lysates of transiently transfected 293T cells (Fig. 1*A*). In addition, endogenous RASSF1A was detected in immunoprecipitates prepared from HeLa cells with antibodies to Mst1 (Fig. 1*A*). To determine the regions of Mst1 and RASSF1A responsible for the association of these two proteins, we generated deletion mutants of each. Deletion of the COOH-terminal 52 amino acids of RASSF1A (RASSF1A-RA mutant, residues 1-288), including the SARAH domain, impaired the interaction of the protein with Mst1, whereas the Mst1(1-430) mutant, which lacks the dimerization domain of the full-length protein, failed to bind to RASSF1A (Fig. 1*B* and *C*). These results thus indicated that Mst1 binds to the SARAH domain in the COOH-terminal region of RASSF1A through a COOH-terminal region that includes its dimerization domain, consistent with the previous findings (17, 18).

Given that RASSF1A localizes to microtubules in a manner dependent on the phase of the cell cycle (29), we examined whether Mst1 colocalizes with RASSF1A throughout the cell cycle by immunofluorescence analysis. A substantial proportion of endogenous Mst1 in HeLa cells localized with endogenous RASSF1A to cytoplasmic microtubules in interphase, to spindle poles and

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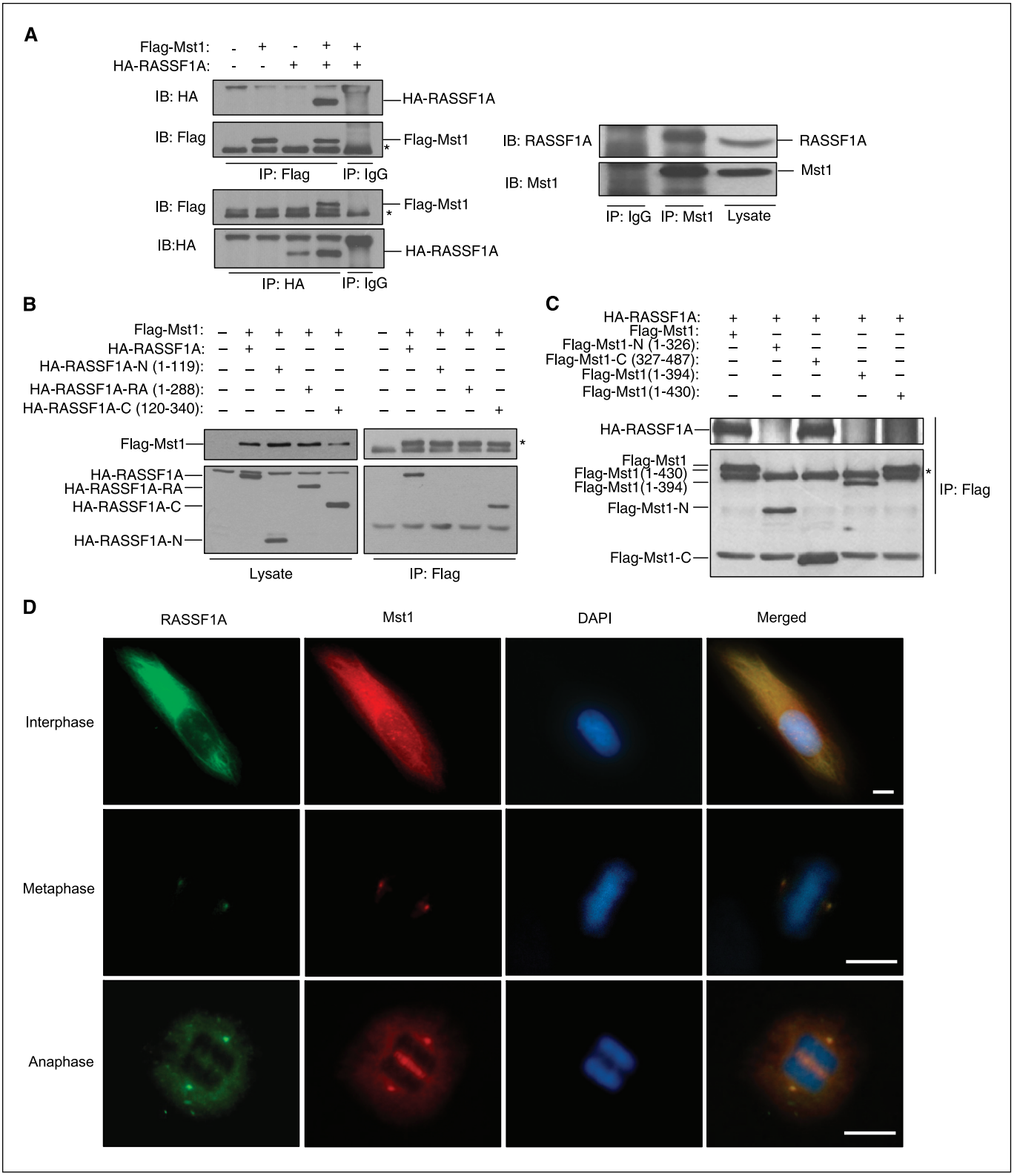

Figure 1. Interaction of Mst1 with RASSF1A in intact cells. *A,* 293T cells were transfected with vectors for Flag-Mst1 and HA-RASSF1A as indicated. Cell lysates were subjected to immunoprecipitation (*IP*) with antibodies to Flag or to HA, and the resulting precipitates were subjected to immunoblot analysis (*IB*) with the same antibodies (*left*). Alternatively, a HeLa cell lysate was subjected to immunoprecipitation with anti-Mst1, and the resulting precipitates as well as the cell lysates were subjected to immunoblot analysis with anti-RASSF1A and anti-Mst1 (*right*). Control immunoprecipitations were done with normal mouse IgG. *, Ig heavy chain. *B,* 293T cells expressing HA-RASSF1A deletion mutants and Flag-Mst1, as indicated, were subjected to immunoprecipitation with anti-Flag, and the resulting precipitates as well as cell lysates were subjected to immunoblot analysis with anti-Flag and anti-HA. *C,* 293T cells expressing Flag-Mst1 deletion constructs and HA-RASSF1A, as indicated, were subjected to immunoprecipitation with anti-Flag and the resulting precipitates were subjected to immunoblot analysis with anti-Flag and anti-HA. *D,* HeLa cells were stained with anti-RASSF1A (*green*), anti-Mst1 (*red*), and DAPI (*blue*). *Yellow color in merged images,* colocalization of RASSF1A and Mst1 in both interphase (*top row*) and mitotic (*bottom two rows*) cells. Scale bars, 10 μm.

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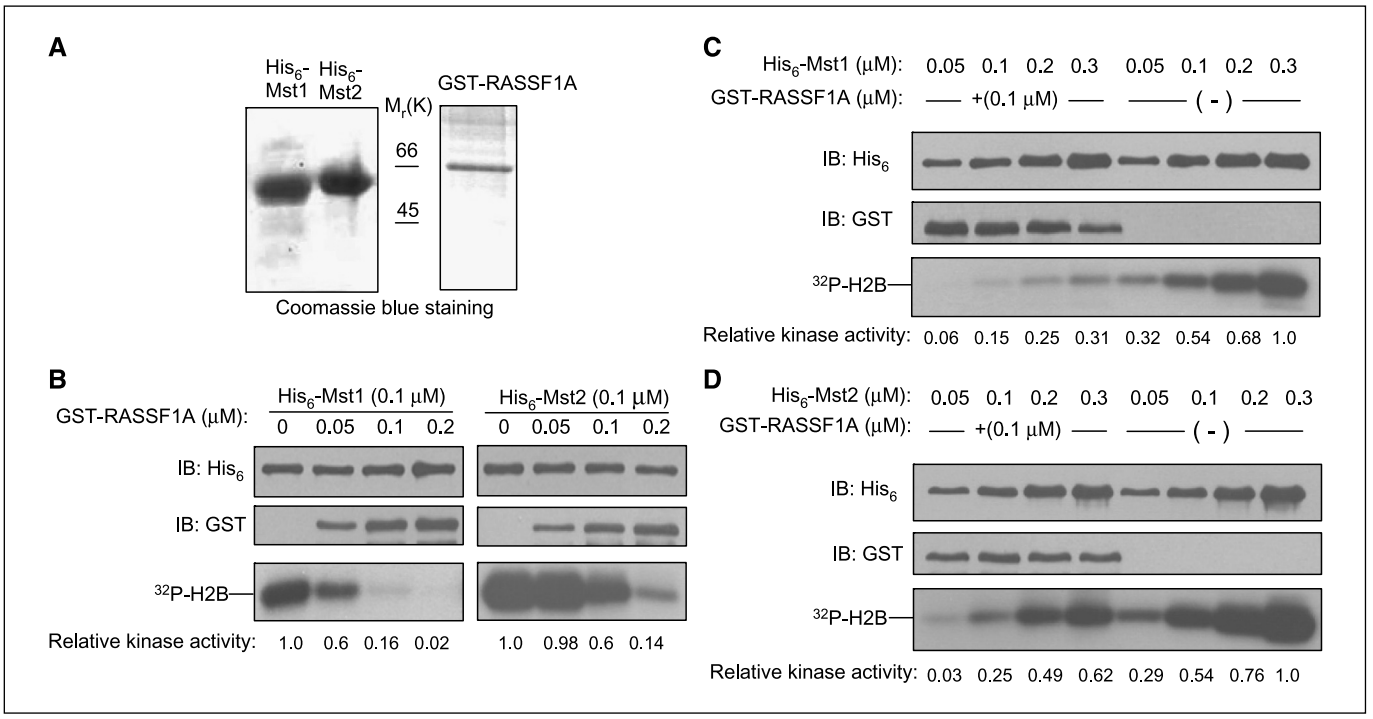

Figure 2. Inhibition of the kinase activity of Mst1/2 *in vitro* by RASSF1A. *A,* the purified fusion proteins of His_6-Mst1, His_6-Mst2, or GST-RASSF1A, used for the kinase assays, were stained with Coomassie blue. *B* to *D,* the kinase activity of the indicated concentrations of purified His_6-Mst1 or His_6-Mst2 was assayed with histone H2B as a substrate and [γ-^{32}P]ATP in the absence or presence of the indicated concentrations of purified GST-RASSF1A. Proteins in the kinase reaction mixture were resolved by SDS-PAGE and then ^{32}P-labeled histone H2B substrates were visualized by autoradiography and the amount of His_6-Mst1/2 and GST-RASSF1A was verified by immunoblotting with antibodies to His_6 tag and to GST, as indicated. Relative kinase activity is indicated below each lane.

associated fibers in metaphase, and to spindle poles and the midzone in anaphase (Fig. 1*D*). In addition, Flag-Mst1 and HA-RASSF1A colocalized in a pattern similar to that evident for the endogenous proteins in transiently transfected HeLa cells (data not shown). These findings further indicate that RASSF1A forms a complex with Mst1 that remains intact throughout the cell cycle.

RASSF1A inhibits the kinase activity of Mst1/2 *in vitro*. We next sought to examine the effect of RASSF1A on the kinase activities of both Mst1 and Mst2 *in vitro*. Human Mst1 and Mst2 share 76% sequence identity, and Mst2 is thought to play a role similar to that of Mst1 in apoptosis (4). For the *in vitro* kinase assay, purified baculovirus-expressed His_6-tagged forms of Mst1 and Mst2 and a bacterially expressed GST-RASSF1A fusion protein were used. A representation of purified proteins stained with Coomassie blue is shown in Fig. 2*A*. In the presence of various amounts of GST-RASSF1A, the kinase activity of recombinant Mst1 or Mst2 toward histone H2B as a substrate was inhibited by GST-RASSF1A in a concentration-dependent manner (Fig. 2*B*). In addition, the kinase activity of various concentrations of His_6-Mst1 or His_6-Mst2 was also inhibited in the presence of GST-RASSF1A (Fig. 2*C* and *D*). These results are consistent with the previous observations that Mst1 kinase activity is inhibited by RASSF1A or NORE1A (18). However, simple inhibition of Mst1 kinase by RASSF1A could not be the actual cellular repertoire in physiologic condition. Indeed, RASSF1A and Hpo, the *Drosophila* homologue of Mst1/2, are both thought to function as tumor suppressors, and RASSF1A might thus have been expected to activate, rather than to inhibit Mst1/2 kinases.

Ectopic expression of RASSF1A increases the kinase activity of Mst1/2 *in vivo*. Considering that *in vivo* observations may provide new insights in understanding of the functional relation of RASSF1A with Mst1 in physiologic condition, we decided to examine the effect of RASSF1A on the kinase activity of Mst1/2 in mammalian cells. We first cotransfected 293T cells with a vector for wild type or a kinase-inactive mutant of Flag-Mst1 together with various amounts of a vector for HA-RASSF1A. Immunoprecipitates prepared from the transfected cells with antibodies to Flag were then assayed for kinase activity with histone H2B as a substrate. The kinase activity of wild-type Mst1 was increased by RASSF1A in a concentration-dependent manner (Fig. 3*A* and *B*), in contrast to the results obtained *in vitro*.

We next examined whether the binding of RASSF1A to Mst1 is required for activation of Mst1 by RASSF1A *in vivo*. Among several conserved residues in the SARAH domain (19), we found that mutation of Leu^{301} of RASSF1A to proline prevented the association of RASSF1A with Mst1 (Fig. 3*C*), indicating that this amino acid is required for binding to Mst1. Expression of HA-tagged RASSF1A(L301P) had only a slight effect on the kinase activity of Flag-Mst1 immunoprecipitated from 293T cells compared with that of wild-type HA-RASSF1A (Fig. 3*C*), indicating that interaction of RASSF1A with Mst1 is required, at least in part, for the effect of the former on the activity of the latter. Expression of HA-RASSF1A also increased the kinase activity of Flag-Mst2 in 293T cells (Fig. 3*D*).

RASSF1A mediates activation of Mst1 during Fas-induced apoptosis. Given that RASSF1A activated Mst1 in transiently transfected mammalian cells, we next investigated whether endogenous RASSF1A mediates endogenous Mst1 activation during the induction of apoptosis. To examine this issue, we generated HeLa cells stably transfected with a vector for RASSF1A siRNA or GFP siRNA as a control, and confirmed the reduction in RASSF1A expression of the cells stably expressing RASSF1A siRNA compared with that of control cells expressing GFP siRNA (Fig. 4*A*). Control and RASSF1A-depleted HeLa cells were treated with antibodies to Fas and then subjected to immunoprecipitation with antibodies to

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Mst1. Assay of the resulting precipitates for kinase activity revealed that the activation of Mst1 by Fas ligation was inhibited in the cells depleted of RASSF1A compared with that apparent in the control cells (Fig. 4*B*), suggesting that RASSF1A is required for maximal activation of Mst1 in response to proapoptotic signaling. The basal kinase activity of Mst1 was also reduced in the RASSF1A-depleted cells.

We next examined the effect of RASSF1A depletion on the apoptosis-specific phosphorylation of histone H2B on Ser^{14} by Mst1, a critical event in cells undergoing apoptosis (8). Immunoblot analysis with antibodies specific for the Ser^{14}-phosphorylated form of histone H2B revealed that the phosphorylation of this residue induced by Fas ligation was rarely detected in HeLa cells depleted of RASSF1A compared with that apparent in control cells (Fig. 4*C*), indicating that the kinase activity of Mst1 toward a physiologic substrate *in vivo* depends on RASSF1A. Moreover, restored expression of RASSF1A in RASSF1A-deficient H1299 cells enhanced the stimulatory effect of Fas ligation on histone H2B phosphorylation on Ser^{14} (Fig. 4*D*). These findings thus suggest that RASSF1A is required for full activation of Mst1 and thereby contributes to the apoptotic function of Mst1.

RASSF1A promotes Mst1-mediated apoptosis. Finally, we examined whether RASSF1A affects Mst1-mediated apoptosis. The TUNEL assay revealed that the incidence of Fas-induced apoptosis in HeLa cells stably depleted of RASSF1A by RNA interference (RNAi) was markedly reduced compared with that apparent in control cells (Fig. 5*A* and *B*). Conversely, stably restored expression of RASSF1A in RASSF1A-deficient A549 cells increased the incidence of Fas-induced apoptosis (Fig. 5*C*), suggesting that RASSF1A stimulates apoptosis in response to Fas-ligation.

To determine whether the effect of RASSF1A on apoptosis was mediated through Mst1, we transfected U2OS cells with a vector for HA-RASSF1A or the corresponding empty vector together with a vector for red fluorescent protein as a marker of transfection. The incidence of Fas-induced apoptosis among transfected cells was about twice greater for those transfected with the HA-RASSF1A vector than for those transfected with the empty vector (Fig. 5*D*). Similar transfection of cells with a vector for HA-RASSF1A(L301P), which does not bind to Mst1, only weakly increased the incidence of Fas-induced apoptosis, consistent with the effect of HA-RASSF1A(L301P) on Mst1 kinase activity (Fig. 5*D*). Furthermore, the potentiating effect of HA-RASSF1A on Fas-induced apoptosis was greatly reduced in U2OS cells stably depleted of Mst1 by RNAi (Fig. 5*E*). These findings thus suggest that RASSF1A regulates apoptosis by promoting the activation of Mst1.

Discussion

The kinase Mst1 and the related protein Mst2 were originally identified as proteins that are activated by certain types of cellular stress (37) and have been shown to participate in a wide range of apoptotic responses (3–9). A *Drosophila* homologue of Mst1/2,

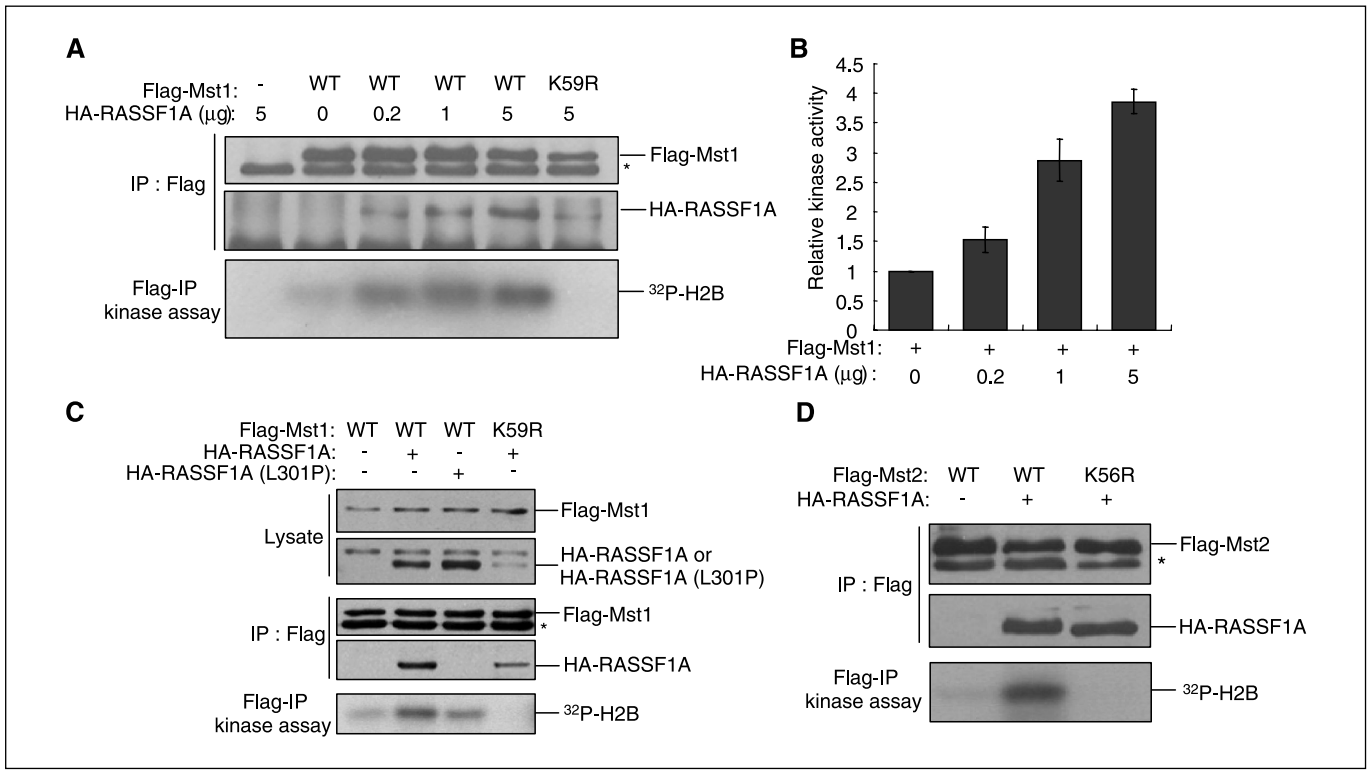

Figure 3. Activation of Mst1/2 by RASSF1A in transfected cells. *A,* 293T cells were cotransfected with a vector for wild-type (*WT*) or the K59R kinase-inactive mutant of Flag-tagged Mst1 (0.2 μg) and various amounts of a vector for HA-RASSF1A. Cell lysates were subjected to immunoprecipitation with anti-Flag and the resulting precipitates were assayed for kinase activity with histone H2B as a substrate and [γ-^{32}P]ATP. The immunoprecipitates were also subjected to immunoblot analysis with anti-Flag and anti-HA. *B,* quantitation of the effect of HA-RASSF1A on Flag-Mst1 activity determined in three independent experiments similar to that shown in (*A*). *Columns,* mean of triplicate assays; *bars,* SE. *C,* lysates from 293T cells cotransfected with vectors for Flag-Mst1 (wild type or the K59R kinase-inactive mutant) and HA-RASSF1A or HA-RASSF1A(L301P), as indicated, were subjected to immunoprecipitation with anti-Flag. The resulting precipitates as well as cell lysates were subjected to immunoblot analysis with anti-Flag and anti-HA. HA-RASSF1A(L301P) did not bind to Flag-Mst1 and the kinase activity of Flag-Mst1 immunoprecipitate was assayed as in (*A*). *D,* lysates from 293T cells transfected with vectors for Flag-Mst2 (wild type or the K56R kinase-inactive mutant) and HA-RASSF1A, as indicated, were subjected to immunoprecipitation with anti-Flag. The resulting precipitates were both assayed for kinase activity and subjected to immunoblot analysis as in (*A*).

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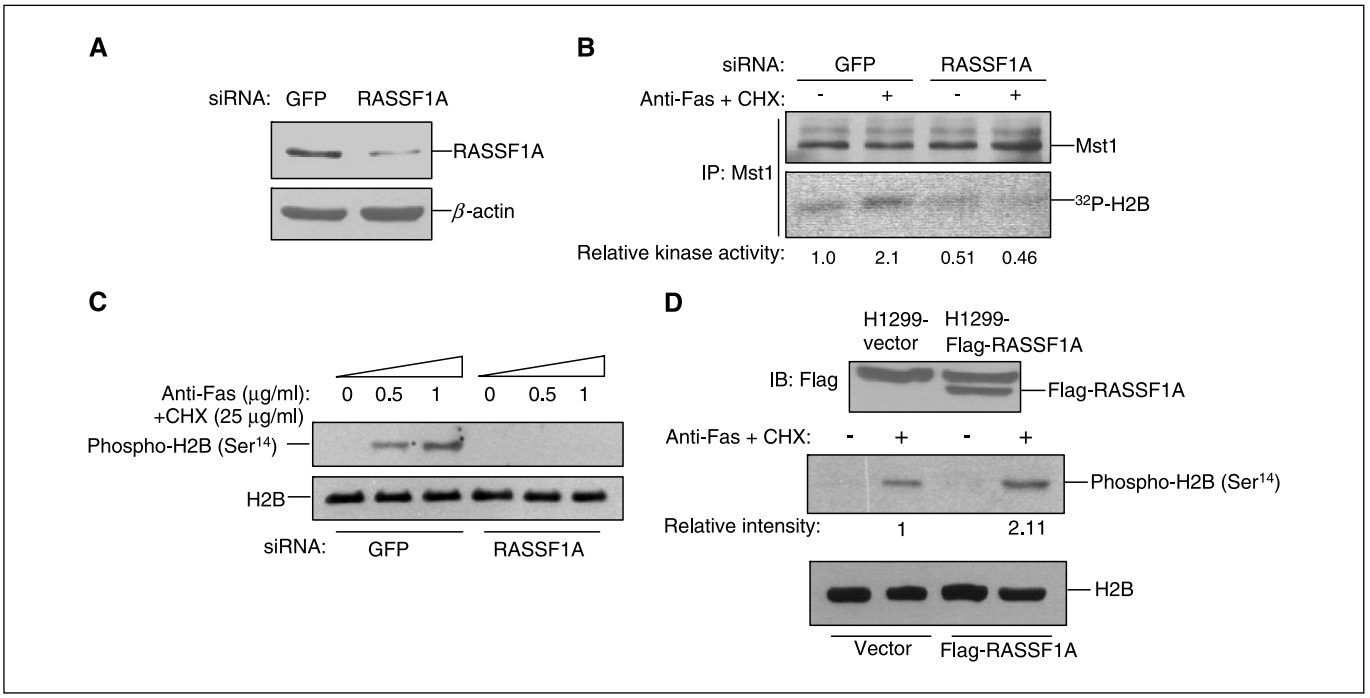

Figure 4. Requirement of RASSF1A for activation of Mst1 during Fas-induced apoptosis. *A,* lysates of HeLa cells stably transfected with a vector for RASSF1A siRNA or GFP siRNA as a control were subjected to immunoblot analysis with anti-RASSF1A and anti-β-actin as a loading control. *B,* control and RASSF1A-depleted cells were either treated with anti-Fas (0.1 μg/mL) for 3 hours or not, cell lysates were prepared and subjected to immunoprecipitation with anti-Mst1, and the resulting precipitates were both assayed for kinase activity with histone H2B as a substrate and subjected to immunoblot analysis with anti-Mst1. Relative kinase activity is indicated below each lane. *C,* control and RASSF1A-depleted HeLa cells were incubated with various amounts of anti-Fas and cycloheximide (*CHX*; 25 μg/mL) for 4 hours, after which the extracts of acid-soluble proteins were prepared from the cells and subjected to immunoblot analysis with antibodies specific for histone H2B phosphorylated on Ser^{14}. The same blot was stripped and reprobed with anti-histone H2B as a loading control. *D,* H1299 cells stably transfected with a retroviral vector for Flag-RASSF1A or with the corresponding empty vector were subjected to immunoblot analysis with anti-Flag (*top*). The cells were also treated with anti-Fas (0.5 μg/mL) and cycloheximide (25 μg/mL) for 13 hours, and acid extracts were prepared from the cells were analyzed as in (*C*). The level of histone H2B phosphorylation on Ser^{14} was quantified and shown as relative intensity (*bottom*).

Hpo, functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis (11–13). Human Mst2 is able to substitute for Hpo in developing *Drosophila* tissues, providing support for a role of Mst as a regulator of cell cycle progression and tumor suppressor in mammalian cells (13). RASSF1A also regulates cell proliferation and functions as a tumor suppressor. It thus regulates mitotic progression by controlling APC-Cdc20 activity (29) and inhibits the G_1-S transition (27). In addition, RASSF1A is thought to participate in Ras-mediated apoptotic signaling (17, 18, 35).

Given the role of Mst1 as an apoptotic kinase and those of RASSF1A and Hpo as tumor suppressors, we hypothesized that RASSF1A and Mst1 act in a common apoptotic pathway. We found that RASSF1A and Mst1 colocalize to microtubules during the cell cycle, suggesting that Mst1 may regulate cell cycle progression in a RASSF1A-dependent or RASSF1A-independent manner. Although RASSF1A inhibits the kinase activity of Mst1 *in vitro*, we here showed that RASSF1A plays a different role *in vivo*, in the activation of the kinase activity of Mst1, which in turn triggers Fas-mediated apoptosis. This implication was supported by our observations, as follows: (*a*) transient overexpression of RASSF1A and Mst1 in mammalian cells resulted in activation of the latter by the former; (*b*) depletion of RASSF1A by RNAi inhibited the activation of Mst1 as well as the phosphorylation of histone H2B (an Mst1 substrate) on Ser^{14} induced by Fas ligation in HeLa cells; (*c*) restored expression of RASSF1A in RASSF1A-deficient cells potentiated the Fas-induced phosphorylation of histone H2B on Ser^{14}. These results suggested that Mst1 activation is largely dependent on RASSF1A in apoptotic cells. Furthermore, depletion or restoration of RASSF1A expression reduced or increased the incidence of apoptosis, respectively, and down-regulation of Mst1 abrogated the potentiation of Fas-induced apoptosis elicited by overexpression of RASSF1A. The potentiation of apoptosis by RASSF1A was thus dependent on Mst1. Together, our findings suggest that RASSF1A mediates the activation of Mst1 in response to apoptotic signaling and thereby promotes Mst1-mediated apoptosis.

However, our observation that the kinase activity of Mst1 was increased by overexpression of RASSF1A *in vivo* is inconsistent with the previous finding that both RASSF1A and NORE1A inhibited the kinase activity of Mst1 *in vivo* (18), although inhibition of Mst1 activity by RASSS1A *in vitro* was observed in both studies. Although it is difficult to find the reasons underlying this discrepancy in the role of RASSF1A in Mst1 activation between two studies, it may be explained by the differences in the level of protein expression, in experimental assay methods for measuring Mst1 kinase activity, or in the signals leading to apoptosis between the studies. In the previous study, the kinase activity of Mst1 seemed to be suppressed in cells expressing an excess of RASSF1A or NORE1A, and Mst1 kinase activity *in vivo* was measured by immunoblotting of Mst1 autophosphorylation on Thr^{183}. In addition, immunoprecipitation-kinase assay was done with the same tagged forms of Mst1 kinase and RASSF1A or NORE1A of which eluted immunoprecipitates could contain an excess of RASSF1A or NORE1A not associated with Mst1. However, in our study, both proteins were expressed with the considered ratio of their amounts similar to the physiologic level and the Mst1 kinase activity was directly measured by immunoprecipitation-kinase assay with immunoprecipitates containing Mst1 and

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RASSF1A present only in a complex. Moreover, whereas the previous study mainly revealed the role of NORE1A in the recruitment of Mst1 to Ras for Mst1 activation, the present study showed the role of RASSF1A in Mst1 activation induced by Fas activation. Thus, NORE1A and RASSF1A may have their own distinct roles in apoptosis in response to specific signals.

Although the effect of RASSF1A on the kinase activity of Mst1 *in vitro* seems inconsistent with that observed *in vivo* in the present study, it is likely that regulation of the kinase activity of Mst1 by RASSF1A in intact cells may be more complex than that mediated by the inhibitory bipartite interaction *in vitro*. It is thus possible that RASSF1A recruits upstream activators or downstream effectors of Mst1 kinase, which potentiates the activation of Mst1. This possibility is further supported by the previous suggestion of Proskova et al. (18) that RASSF1A or NORE1A could maintain the low basal activity of Mst1, but mediate some activating inputs, given that Ras-NORE1 complex ultimately provides Mst1 activation. In this manner, RASSF1A may have a dual role that could switch itself between an inhibitor and an activator, depending on the specific cellular context, particularly in the presence of apoptotic signals. Alternatively, RASSF1A and Mst1 may form a complex with other unidentified proteins that affect the regulation of the latter by the former, with the stimulatory effect of RASSF1A on the kinase activity of Mst1 being apparent only in the presence of these additional proteins. Purified RASSF1A proteins may thus simply inhibit purified Mst1 kinase activity in the absence of these additional factors. Therefore, it will be important to identify the proteins that affect the RASSF1A-mediated Mst1 activation in apoptotic signaling. Of interest, it has been shown that CNK1 associates with RASSF1A-Mst1 complex and participates in the proapoptotic signaling initiated by active Ras (34). It was also noted that hWW45

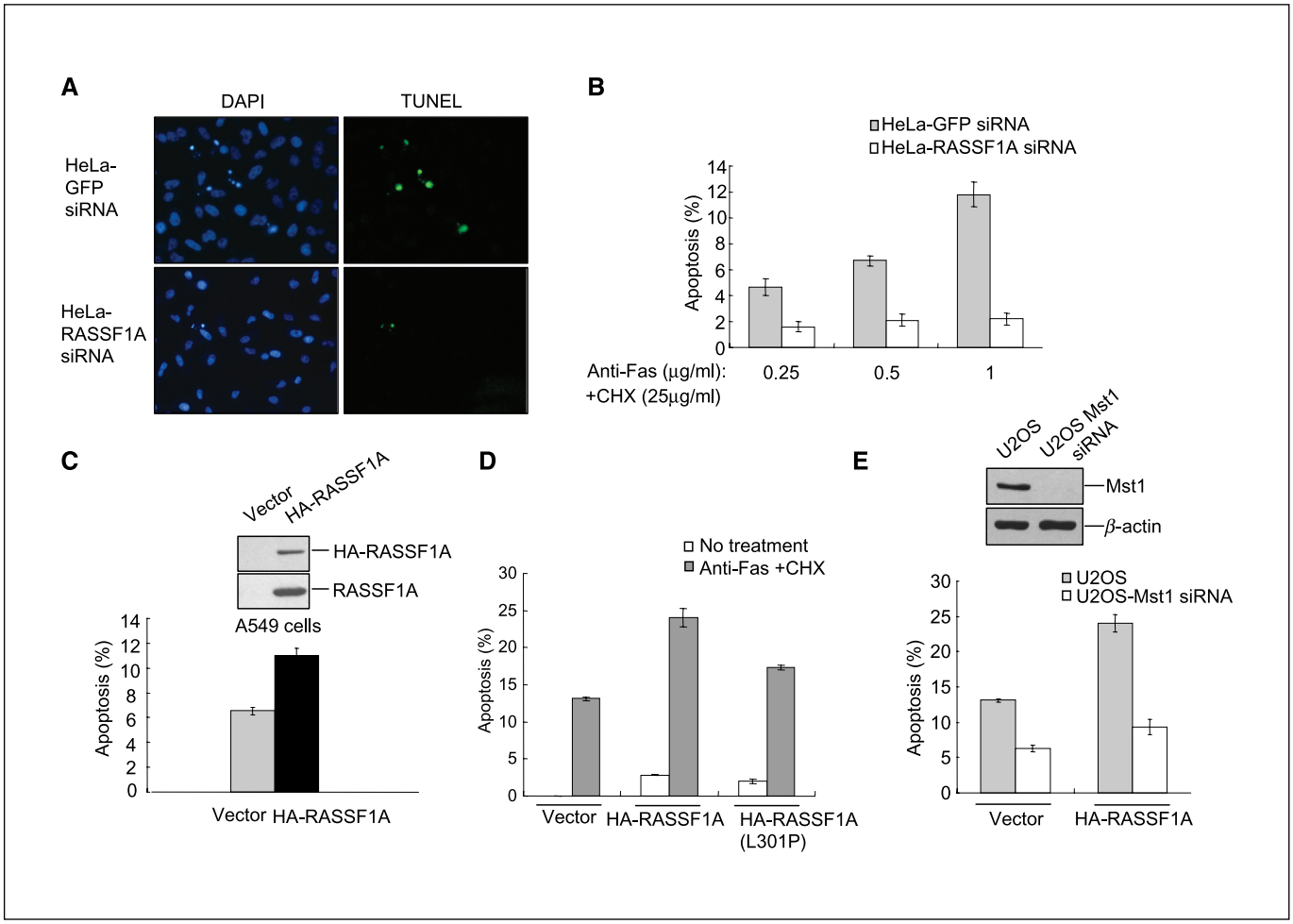

Figure 5. Promotion by RASSF1A of Mst1-mediated apoptosis. *A,* control and RASSF1A siRNA-expressing HeLa cells were treated for 4 hours with anti-Fas (0.5 μg/mL) and cycloheximide (25 μg/mL) and were then stained with DAPI and by the TUNEL protocol. TUNEL-positive cells (*green*) exhibit condensed or fragmented DAPI-stained nuclei (*blue*), indicative of apoptosis. *B,* control and RASSF1A-depleted HeLa cells were incubated for 4 hours with the indicated concentrations of anti-Fas and cycloheximide (25 μg/mL), after which the percentage of TUNEL-positive cells was determined. *Columns,* mean of three experiments; *bars,* SE. *C,* lysates of A549 cells stably transfected with a retroviral vector for HA-RASSF1A or with the corresponding empty vector were subjected to immunoblot analysis with anti-HA and anti-RASSF1A (*top*). The cells were also treated with anti-Fas (2 μg/mL) and cycloheximide (50 μg/mL) for 4 hours, after which the percentage of TUNEL-positive cells was determined. *Columns,* mean of three experiments; *bars,* SE (*bottom*). *D,* U2OS cells were cotransfected with a vector for HA-RASSF1A or HA-RASSF1A(L301P) or with the corresponding empty vector together with a vector for red fluorescent protein as a marker of transfection. After 48 hours, the cells were incubated for 4 hours in the absence or presence of anti-Fas (0.1 μg/mL) and cycloheximide (25 μg/mL), and the percentage of TUNEL-positive cells among red fluorescent protein–positive cells was determined. *Columns,* mean of three experiments; *bars,* SE. *E,* lysates of U2OS cells or those stably transfected with a vector for Mst1 siRNA were subjected to immunoblot analysis with anti-Mst1 and anti-β-actin as a loading control (*top*). Control and Mst1-depleted U2OS cells were also cotransfected with a vector for HA-RASSF1A or the corresponding empty vector together with a vector for red fluorescent protein. The induction of apoptosis and consequent analysis were done as in (*D, bottom*).

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associates with RASSF1A-Mst1, which could thereby converge in a common pathway through SARAH domain (19, 20). Thus, CNK1 or hWW45 may be one of unknown factors for RASSF1A-mediated Mst1 activation in response to apoptotic signals. Importantly, the work using *in vitro* system may preclude the understanding of the regulation of Mst1 in the cellular context where associated signaling molecules might provide complex environment in response to specific signals. Thus, our observations that depletion of RASSF1A inhibited activation of Mst1 as well as the phosphorylation of H2B on Ser^{14} induced by Fas ligation may provide more substantial evidence for Mst1 activation by RASSF1A *in vivo*.

It was recently shown that Raf-1 negatively regulates the kinase activity of Mst2 during apoptotic signaling by inhibiting the phosphorylation of Mst2 that is required for its activation (38). The binding of Raf-1 to Mst2, which is sufficient to inhibit the kinase activity of the latter, induces the dissociation of Mst2 homodimers and recruits a phosphatase, resulting in inhibited Mst2 phosphorylation at the critical residues in the activation loop (38). The relief of Raf-1-mediated inhibition may not be sufficient, however, for full activation of Mst2 in response to apoptotic stimuli, given that dissociation of the Mst2-Raf-1 complex in response to growth factor signaling induces only limited Mst2 activation (38). We have shown that RASSF1A interacts with and stimulates the kinase activity of Mst2 in transiently transfected mammalian cells (Fig. 2*D*). Association of RASSF1A with Mst1/2 may thus be an important determinant of maximal activation of these kinases that released from Raf-1-mediated inhibition during apoptotic signaling. Recently, association of RASSF1A with the BH3-like protein modulator of apoptosis-1 (MAP-1) was shown to be induced by death receptor signaling and this interaction was required for a conformational change of the proapoptotic protein Bax and maximal apoptosis (39). It will thus be important to examine whether this RASSF1A-MAP-1 pathway is also mediated by Mst1. In addition, the precise molecular mechanism by which RASSF1A activates Mst1 and its downstream effectors remains to be determined.

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