The RASSF1A tumor suppressor protein interacts with the pro-apoptotic mammalian STE20-like kinases MST1 and MST2 and induces their autophosphorylation and activation, but the mechanism of how RASSF1A activates MST1/2 is unclear. Okadaic acid treatment and PP2A knockdown promoted MST1/2 phosphorylation. Data from dephosphorylation assays and reduced activation of MST1/2 seen after RASSF1A depletion suggest that dephosphorylation of MST1/2 on Thr-183 and Thr-180 by PP2A is prevented by RASSF1A, shifting the balance of MST1/2 to the activated autophosphorylated form. In addition to preventing dephosphorylation, RASSF1A also stabilized the MST2 protein. Through binding to MST1/2, RASSF1A supports maintenance of MST1/2 phosphorylation, promoting an active state of the MST kinases and favoring induction of apoptosis. This is one of the first examples of a tumor suppressor acting as an inhibitor of a specific dephosphorylation pathway.

The RASSF1A (Ras association domain family 1 isoform A) gene is localized on chromosome 3p21.3, in an area that likely harbors at least one important tumor suppressor gene (1, 2). RASSF1A is frequently silenced by promoter hypermethylation in many human tumors (3, 4). Rassf1a-targeted mice are prone to spontaneous and carcinogen-induced tumorigenesis (5, 6). The RASSF1A protein is involved in several growth regulatory and pro-apoptotic pathways. Recent work has shown that RASSF1A associates with components of the mammalian Hippo signaling network (7), an emerging pathway implicated in organ size control, restriction of cell proliferation, apoptosis, and tumor suppression (8–10). RASSF1A interacts with the mammalian kinases MST1 and MST2, the orthologs of the Drosophila Hippo kinase, and activates MST1 and MST2 by promoting their autophosphorylation and phosphorylation of the downstream LATS1 kinase (7).

The mammalian Sterile-20-like kinases MST1 (also known as STK4 and KRS2) and MST2 (also known as STK3 and KRS1) belong to the class II germinal center (Ser/Thr protein) kinases (11). MST1 and MST2 have recently been implicated as important tumor suppressors (12–14), suggesting that the RASSF-MST complexes may represent intriguing tumor-suppressing modules. Besides their potential role in promoting apoptosis through the Hippo pathway, MST1 and MST2 are implicated in several other pro-apoptotic processes. During induction of apoptosis, MST1 and MST2 can be activated, leading to phosphorylation of histone H2B and nuclear DNA fragmentation (15). In addition, the activation of JNK (Jun N-terminal kinase) signaling (16, 17) and phosphorylation of FOXO3 transcription factors (18) have also been associated with MST-induced apoptosis.

The MST1 and MST2 kinases are regulated by several mechanisms, including phosphorylation, caspase cleavage, dimerization, and cofactors (19). They are activated in response to staurosporine (STS), a potent protein kinase C inhibitor and apoptosis inducer, or the protein phosphatase inhibitor okadaic acid (20). Several other pro-apoptotic stimuli and stresses have also been reported to induce MST1/2 kinase activity, including Fas ligand, TNFα, H2O2, serum starvation, and UV irradiation (19), but not cytokines, growth factors, protein synthesis inhibitors, DNA-damaging agents, protein denaturants, and forskolin (21). MST1 and MST2 share 78% identity and contain an N-terminal catalytic domain and a C-terminal SARAH (Salvador-Rassf-Hippo) domain known to be involved in homo- and heterodimerization reactions (22). Caspase cleavage at a caspase motif (MST1, DEMD326; and MST2, DELD322) can release a truncated N-terminal MST cleavage product that has increased activity and translocates to the nucleus, where it promotes phosphorylation of Ser-14 on histone H2B (15, 23–25). However, MST1/2 and their caspase-insensitive mutants can be activated by STS, which can induce MST activation prior to cleavage at an early time point of treatment (25). Caspase-mediated cleavage is neither required nor sufficient to activate MST1/2, although it is often coupled to MST activation. Existing data suggest that full activation of MST1 during apoptosis requires both phosphorylation and proteolysis (17, 26).

Autophosphorylation of MST1 Thr-183 and MST2 Thr-180 within the activation loop is critical for kinase activity (17, 23, 26). Protein phosphatase treatment markedly reduces MST1 activity in anti-Fas antibody-treated cells (23). Okadaic acid and calyculin A, inhibitors of both type 1 and type 2A protein phosphatases, stimulate MST1/2 activation, which indicates that MST1 and MST2 are maintained unphosphorylated by protein phosphatases in the absence of stressors (20, 21). Okadaic acid and other protein phosphatase inhibitors stabilize MST1/2, suggesting that dephosphorylation of MST1/2 is required for activation (20). These studies, when combined, indicate that dephosphorylation of MST1/2 is required for their induction of apoptosis. 

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

1 To whom correspondence should be addressed. Tel.: 626-301-8853; Fax: 626-358-7703; E-mail: gpfeifer@coh.org.

2 The abbreviation used is: STS, staurosporine.
RASSF1A and MST Kinases

27). However, it is unclear which specific phosphatase(s) is responsible for MST1/2 dephosphorylation in vitro and in vivo (23, 26).

RASSF1A is known to promote MST1/2 autophosphorylation and activation (7, 28–30). However, it still remains unclear how RASSF1A can activate MST kinases. Here, we demonstrate that RASSF1A activates MST1 and MST2 by preventing their dephosphorylation.

EXPERIMENTAL PROCEDURES

Materials—pCMV-FLAG-MST1 and pCMV-FLAG-MST2 (wild-type and kinase-dead) have been described previously (7). pCMV-HA-RASSF1A was kindly provided by Dr. A. Khokhlatchev (University of Virginia). The PP2A α-isoform catalytic subunit (PPP2CA) was cloned into the pcDNA3.0–3HA vector. Anti-RASSF1A antibody (M304) has been described previously (7). Anti-RASSF1A monoclonal (clone 3F3) was purchased from Abcam (Cambridge, MA). Anti-phospho-MST antibody was kindly provided by Dr. J. Avruch (Massachusetts General Hospital). Anti-MST2, anti-LATS1, and anti-HA antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Anti-MST1 and anti-PP2Ac (05–421) antibodies were purchased from Upstate (Lake Placid, NY). Anti-PP1α antibody (AB4082) was from Chemicon International (Temecula, CA). Anti-phospho-Thr, anti-cleaved caspase-3 (9661), anti-poly(ADP-ribose) polymerase (9532), anti-phospho-YAP Ser-127 (4911), anti-p38 (9212), anti-phospho-p38 (Thr-180/Tyr-182) (9211), anti-AKT (sc-292), anti-phospho-AKT (Ser-473) (193H12, 4058), anti-p44/p42 ERK1/2 (137F5, 4695), and anti-phospho-p44/p42 ERK1/2 (Thr-202/Tyr-204) (9101) antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti-JNK (sc-9711), mouse anti-phospho-JNK (9H8, sc-81502), anti-YAP, and anti-HDAC1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin antibody (clone TUB 2.1), anti-β-actin antibody (clone AC-15), and EZView™ Red anti-FLAG M2 affinity gel were purchased from Sigma. Anti-FLAG M2 was from Stratagene. PP1 was from New England Biolabs (Ipswich, MA). PP2A–isoform catalytic subunit) were purchased from Integrated DNA Technologies. RASSF1A siRNA 196 has been described previously (31).

Materials—pCMV-FLAG-MST1 and pCMV-FLAG-MST2 (wild-type and kinase-dead) have been described previously (7). pCMV-HA-RASSF1A was kindly provided by Dr. A. Khokhlatchev (University of Virginia). The PP2A α-isoform catalytic subunit (PPP2CA) was cloned into the pcDNA3.0–3HA vector. Anti-RASSF1A antibody (M304) has been described previously (7). Anti-RASSF1A monoclonal (clone 3F3) was purchased from Abcam (Cambridge, MA). Anti-phospho-MST antibody was kindly provided by Dr. J. Avruch (Massachusetts General Hospital). Anti-MST2, anti-LATS1, and anti-HA antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Anti-MST1 and anti-PP2Ac (05–421) antibodies were purchased from Upstate (Lake Placid, NY). Anti-PP1α antibody (AB4082) was from Chemicon International (Temecula, CA). Anti-phospho-Thr, anti-cleaved caspase-3 (9661), anti-poly(ADP-ribose) polymerase (9532), anti-phospho-YAP Ser-127 (4911), anti-p38 (9212), anti-phospho-p38 (Thr-180/Tyr-182) (9211), anti-AKT (sc-292), anti-phospho-AKT (Ser-473) (193H12, 4058), anti-p44/p42 ERK1/2 (137F5, 4695), and anti-phospho-p44/p42 ERK1/2 (Thr-202/Tyr-204) (9101) antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti-JNK (sc-9711), mouse anti-phospho-JNK (9H8, sc-81502), anti-YAP, and anti-HDAC1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin antibody (clone TUB 2.1), anti-β-actin antibody (clone AC-15), and EZView™ Red anti-FLAG M2 affinity gel were purchased from Sigma. Anti-FLAG M2 was from Stratagene. PP1 was from New England Biolabs (Ipswich, MA). PP2A–isoform catalytic subunit) were purchased from Integrated DNA Technologies. RASSF1A siRNA 196 has been described previously (31). The siRNA oligonucleotides were delivered into HeLa cells using Lipofectamine 2000 (Invitrogen).

Materials—pCMV-FLAG-MST1 and pCMV-FLAG-MST2 (wild-type and kinase-dead) have been described previously (7). pCMV-HA-RASSF1A was kindly provided by Dr. A. Khokhlatchev (University of Virginia). The PP2A α-isoform catalytic subunit (PPP2CA) was cloned into the pcDNA3.0–3HA vector. Anti-RASSF1A antibody (M304) has been described previously (7). Anti-RASSF1A monoclonal (clone 3F3) was purchased from Abcam (Cambridge, MA). Anti-phospho-MST antibody was kindly provided by Dr. J. Avruch (Massachusetts General Hospital). Anti-MST2, anti-LATS1, and anti-HA antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Anti-MST1 and anti-PP2Ac (05–421) antibodies were purchased from Upstate (Lake Placid, NY). Anti-PP1α antibody (AB4082) was from Chemicon International (Temecula, CA). Anti-phospho-Thr, anti-cleaved caspase-3 (9661), anti-poly(ADP-ribose) polymerase (9532), anti-phospho-YAP Ser-127 (4911), anti-p38 (9212), anti-phospho-p38 (Thr-180/Tyr-182) (9211), anti-AKT (sc-292), anti-phospho-AKT (Ser-473) (193H12, 4058), anti-p44/p42 ERK1/2 (137F5, 4695), and anti-phospho-p44/p42 ERK1/2 (Thr-202/Tyr-204) (9101) antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti-JNK (sc-9711), mouse anti-phospho-JNK (9H8, sc-81502), anti-YAP, and anti-HDAC1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin antibody (clone TUB 2.1), anti-β-actin antibody (clone AC-15), and EZView™ Red anti-FLAG M2 affinity gel were purchased from Sigma. Anti-FLAG M2 was from Stratagene. PP1 was from New England Biolabs (Ipswich, MA). PP2A–isoform catalytic subunit) were purchased from Integrated DNA Technologies. RASSF1A siRNA 196 has been described previously (31). The siRNA oligonucleotides were delivered into HeLa cells using Lipofectamine 2000 (Invitrogen).
RESULTS

RASSF1A-associated MST1 and MST2 Kinases Are Active—RASSF1A interacts with MST1 and MST2 kinases (7, 28–30, 32, 33). When endogenous RASSF1A was immunoprecipitated from HeLa cells and coprecipitating proteins were analyzed by mass spectrometry, we identified MST1 and MST2 as two of the three major RASSF1A-interacting proteins (Qscore of 82.9 with 214 of 487 amino acids covered for MST1 and Qscore of 95 with 224 of 491 amino acids covered for MST2) (data not shown), confirming the specificity and strength of this interaction. We conducted in vitro kinase assays with MST1 and MST2 in the absence and presence of cotransfected RASSF1A. As shown in supplemental Fig. S1A, wild-type MST1 and MST2, but not the kinase-dead forms of these proteins, underwent substantial autophosphorylation and also phosphorylated HA-RASSF1A, indicating that RASSF1A-associated MST1 and MST2 kinases are active. We also performed anti-phospho-Thr blotting with the anti-HA-RASSF1A precipitates. Again, phosphorylated HA-RASSF1A was detected only when wild-type FLAG-MST1/2 was coexpressed (supplemental Fig. S1B, left panel). Consistent with our previous results (7), the levels of Thr-phosphorylated MST1/2 were increased by RASSF1A (supplemental Fig. S1B, right panels). The level of activation by RASSF1A is usually considerably stronger for MST1 than for MST2 because of higher basal levels of MST2 phosphorylation (34). C-terminal SARAH domain-deleted RASSF1A cannot promote MST phosphorylation because of lack of interaction of the two proteins (7, 28, 32).

RASSF1A Prevents MST1/2 Dephosphorylation—MST1/2 phosphorylation is enhanced by RASSF1A, requiring the C-terminal SAHAH domain of RASSF1A for interaction and stimulation (7). Therefore, it seemed plausible that RASSF1A might counteract the role of MST1/2 phosphatases. To test this hypothesis, we performed in vitro dephosphorylation assays to determine whether RASSF1A could prevent MST1/2 dephosphorylation. MST phosphorylation was scored with a phospho-specific MST1 Thr-183 and MST2 Thr-180 antibody (34). As shown in Fig. 1, recombinant PP1 and recombinant PP2A phosphatases could dephosphorylate immunoprecipitated MST1/2 in vitro, and this dephosphorylation was partially to completely inhibited by RASSF1A. Importantly, the -fold increase of RASSF1A-induced MST1/2 phosphorylation was much more pronounced with the addition of PP1 (Fig. 1A, upper panel) or PP2A (Fig. 1B, upper panel), suggesting that RASSF1A could counteract the role of phosphatases and prevent MST1/2 dephosphorylation.

MST1/2 can be activated by okadaic acid, which is a cell-permeable phosphatase inhibitor (20, 21). Okadaic acid has the strongest inhibitory effect on PP2A, followed by PP1. Using the phosho-specific MST1 Thr-183 and MST2 Thr-180 antibodies (34), we confirmed that okadaic acid induces MST1/2 Thr-183/Thr-180 phosphorylation (Fig. 2A). This antibody recognizes equivalent phosphorylation sites on both kinases. Because of their almost identical size, MST1 and MST2 cannot usually be resolved on SDS gels, and the phospho-specific antibody did not give reliable signals on immunoprecipitated MST proteins; therefore, phosphorylation of endogenous MST1/2 is scored together. To further investigate which phosphatase is responsible for MST1/2 dephosphorylation, we used siRNA to knock down the catalytic subunit of the PP2A α-isofrom and used pan-PP1 siRNA to knock down the α-, β-, and γ-isofroms of PP1 in STS-treated HeLa cells. Treatment of HeLa cells with STS leads to an increase in phospho-MST levels and induction of apoptosis as indicated by cleavage of poly(ADP-ribose) polymerase (Fig. 2B). We quantitated phospho-MST levels and normalized these to total MST1/2 protein levels. From the combined data of several independent experiments, PP1 siRNA knockdown only marginally increased MST1 Thr-183 or MST2 Thr-180 phosphorylation, but PP2A siRNA did so effectively, indicating that PP2A is a major phosphatase for MST1/2 dephosphorylation in vivo (Fig. 2, B and C). PP2A knockdown enhanced phospho-MST levels also in non-STS-treated HeLa cells, but PP1 knockdown did not (see below, Fig. 5A).

We then transfected FLAG-MST1/2 with HA-RASSF1A and HA-PPP2CA (the PP2A α-isofrom catalytic subunit) into 293T cells. FLAG-MST1/2 was partially dephosphorylated by...
PPP2CA, but the dephosphorylation was almost completely prevented when RASSF1A was coexpressed (Fig. 2D). The results raise the question of whether the phosphatase can form a complex with MST and/or RASSF1A proteins. A weak association of PP2A with FLAG-tagged MST2 was observed and was slightly reduced after cotransfection of RASSF1A (Fig. 2D). Somewhat surprisingly, MST1 showed more association with PP2A in the presence of overexpressed RASSF1A, perhaps indicating the existence of a complex poised for MST1 dephosphorylation once RASSF1A is removed from the complex. However, we were unable to detect an association of endogenous PP2A with endogenous RASSF1A or endogenous MST2.
RASSF1A and MST Kinases

(data not shown). These complexes are probably too transient to be detected by immunoprecipitation.

**RASSF1A Deficiency, MST1/2 Stability, and Activation**—Because RASSF1A and MST1/2 form tight complexes, we tested if the lack of RASSF1A, a phenomenon commonly observed in human tumors, leads to changes in the levels of MST1 and MST2 proteins. After knockdown of RASSF1A in HeLa cells, we observed that the levels of MST2 were reduced to some extent, but MST1 levels were not much affected (Fig. 3, A and B). This indicates that MST2 stability is slightly compromised in RASSF1A-deficient cells. On the other hand, knockdown of RASSF1A led to a substantial reduction in levels of MST1/2 phosphorylation, only partially attributable to the effects on total levels of MST proteins (Fig. 3, A and C). We also did cotransfection experiments to determine the effects of RASSF1A on MST protein levels. Coexpression of RASSF1A did not increase the levels of MST1 or MST2 (data not shown).

If RASSF1A protects MST1/2 from dephosphorylation by PP2A, a treatment with siRNAs directed against RASSF1A and PP2A should result in no change to the level of phospho-MST and apoptosis induced by STS. This is indeed the case (supplemental Fig. S2).

To test if the effect of RASSF1A is specific for preventing dephosphorylation of phospho-MST, we tested the phosphorylation status of other PP2A target proteins in RASSF1A-depleted cells. As shown in Fig. 3D, the phosphorylation of ERK1/2, AKT, and JNK was not notably diminished upon RASSF1A depletion, both in untreated cells and in STS-treated cells. The levels of total p38 and phospho-p38 were reduced by a similar extent upon RASSF1A depletion after STS treatment. (When normalized to total p38, the phospho-p38 level upon RASSF1A knockdown was 1.06 ± 0.04 times that of control siRNA.) Phospho-ERK1/2 was increased in untreated RASSF1A-depleted cells. Therefore, the function of RASSF1A in preventing MST dephosphorylation is a specific event because of direct interaction between the two proteins.

**MST-RASSF1A Complexes during Apoptosis**—The effect of RASSF1A depletion on apoptosis was tested by treating HeLa cells with the apoptotic inducer STS and scoring cleaved caspase-3-positive cells at different time points following treatment. As shown in Fig. 4 (A and B), knockdown of RASSF1A led to a reduction of the percentage of apoptotic cells 2 h after treatment, which is in line with the reduced MST activation seen following RASSF1A depletion (Fig. 3) and is also in agreement with previous data (29). Apoptosis indicated by caspase-3 cleavage was reduced to a similar level by knockdown of MST2 (Fig. 4, A and B), but decreased caspase-3 cleavage was not observed after depleting MST1 or SAV1 (data not shown).

The level of RASSF1A protein was reduced in the presence of MST2 siRNA (Fig. 4C). The most likely explanation is that the RASSF1A protein is stabilized by MST2. This is often found when one protein in a complex enhances the stability of other complex components, but it may also be due to phosphorylation of RASSF1A by MST2 (supplemental Fig. S1), making the RASSF1A protein more stable, a possibility that will require further investigation.

RASSF1A interacts with and activates MST1/2 kinases by preventing their dephosphorylation. However, it still remained unclear whether pro-apoptotic stimuli can induce or enhance the interaction between RASSF1A and MST1/2, leading to MST1/2 activation. We treated HeLa cells with STS and performed Western blotting and co-immunoprecipitation assays on cell lysates (Fig. 4, D–G). RASSF1A was evenly immunoprecipitated by the M304 antibody with moderately decreasing levels in the lysates after treatment (Fig. 4D). The levels of MST1 and MST2 were strongly reduced 2 h after treatment. Similar or even slightly higher levels of MST1/2 were reduced in anti-RASSF1A immunoprecipitates after 0.5- and 1-h treatments with STS when total levels of MST1/2 were reduced in the extracts (Fig. 4, D and G). When we used anti-MST1 or anti-MST2 antibodies to immunoprecipitate MST complexes (Fig. 4, E and F), the levels of coprecipitated RASSF1A were constant but increased relative to the levels of immunoprecipitated MST proteins toward the 2-h time point. Importantly, these findings indicate that most of the RASSF1A-MST1/2 complexes are preformed before induction of a pro-apoptotic stimulus (see also supplemental Fig. S3). Because the total levels of full-length MST1/2 and RASSF1A were all reduced (more strongly for MST1/2) (Fig. 4D) whereas the amount of RASSF1A stayed constant in the MST1/2 immunoprecipitated products (Fig. 4, E and F), the ratio of MST proteins that form a complex with RASSF1A relative to free MST appears to be increased after STS treatment, suggesting that these complexes are particularly stable.

**Signaling to the Hippo Pathway**—Our investigations on MST activation were conducted within the context of STS-induced apoptosis. Because one function of the mammalian MST kinases may be within the Hippo-Salvador-Warts pathway, we have examined the final destination of Hippo signaling by looking at YAP (Yes-associated protein) phosphorylation and intracellular localization of YAP when apoptosis is induced by STS. Unexpectedly, we observed that YAP phosphorylation was strongly decreased rather than increased after STS treatment, and the cytoplasmic-to-nuclear ratio was also decreased (Fig. 5). The reason for this might be the dramatically decreased levels of LATS1, a YAP kinase downstream of MST, seen after STS-induced apoptosis (Fig. 5). Similarly, and also unexpectedly, phosphatase knockdown, which was accompanied by apoptosis, also strongly reduced (rather than increased) YAP phosphorylation and reduced the levels of total YAP protein, again perhaps attributable to depressed levels of LATS1 under these conditions (Fig. 5). It is likely that STS-induced apoptosis and apoptosis induced by inhibition of phosphatases do not operate through the Hippo pathway targeting YAP but rather involve other MST kinase-engaged pathways such as phosphorylation of histone H2B or phosphorylation of FOXO3 (19). Likewise, the data do not exclude the possibility that RASSF1A/MST signaling to YAP is crucial under conditions when the Hippo pathway is activated by more physiological stimuli, such as death receptor stimulation (28–30).
RASSF1A and MST Kinases

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LacZ siRNA</th>
<th>RASSF1A siRNA</th>
<th>RASSF1A siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td><img src="image" alt="IB: anti-P-MST" /></td>
<td><img src="image" alt="IB: anti-MST2" /></td>
<td><img src="image" alt="IB: anti-MST1" /></td>
</tr>
<tr>
<td>STS 1h</td>
<td><img src="image" alt="IB: anti-RASSF1A" /></td>
<td><img src="image" alt="IB: anti-β-actin" /></td>
<td></td>
</tr>
</tbody>
</table>

B

MST1/2 stability in untreated HeLa cells

- MST2: [P<0.05]
- MST1: [P<0.05]

![Graph showing MST1/2 protein level](image)

- LacZ siRNA
- RASSF1A siRNA

C

P-MST1/2 in 1h STS-treated HeLa cells

- Unnormalized
- Normalized to MST2
- Normalized to MST1

![Graph showing P-MST1/2 levels](image)

- LacZ siRNA
- RASSF1A siRNA

D

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LacZ siRNA</th>
<th>RASSF1A siRNA</th>
<th>RASSF1A siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td><img src="image" alt="IB: JNK" /></td>
<td><img src="image" alt="IB: ERK1/2" /></td>
<td><img src="image" alt="IB: AKT" /></td>
</tr>
<tr>
<td>STS 1h</td>
<td>p-JNK</td>
<td>p-ERK1/2</td>
<td>p-S473-AKT</td>
</tr>
<tr>
<td>LacZ siRNA</td>
<td><img src="image" alt="IB: p38" /></td>
<td><img src="image" alt="IB: RASSF1A" /></td>
<td><img src="image" alt="IB: RASSF1A" /></td>
</tr>
<tr>
<td>RASSF1A siRNA</td>
<td><img src="image" alt="IB: p-p38" /></td>
<td><img src="image" alt="IB: β-tub" /></td>
<td><img src="image" alt="IB: β-tub" /></td>
</tr>
</tbody>
</table>

- JNK
- p-JNK
- p38
- p-p38
- RASSF1A
- β-tub
DISCUSSION

Several studies have shown that RASSF1A promotes MST1/2 phosphorylation and activation (7, 28–30), but the detailed mechanisms of this activation are unclear. Curiously, the RASSF1A and the related RASSF5 (NORE1) polypeptides inhibited MST activation when added in vitro, i.e. in the absence of phosphatases (21, 28). Our data demonstrating that RASSF1A counteracts dephosphorylation of MST1/2, thus leading to activation, provide at least a partial explanation for this apparent discrepancy. Okadaic acid, a Ser/Thr protein phosphatase inhibitor, induces MST1/2 activation. Both PP1 and PP2A could dephosphorylate MST1/2 in vitro (Fig. 1), but PP2A siRNA knockdown induced higher levels of MST1/2 phosphorylation than PP1 siRNA knockdown (Fig. 2). These results were quantitated from three independent experiments, and S.D. is shown.

FIGURE 3. Effect of RASSF1A depletion on MST1/2 protein levels and phosphorylation and on other phosphoproteins. A, RASSF1A-specific siRNA was used in control and STS-treated HeLa cells to diminish RASSF1A levels, and the effect of the depletion on MST1 and MST2 protein levels and their Thr-183/Thr-180 phosphorylation status was assessed. The asterisk indicates a nonspecific band. B, depletion of RASSF1A leads to a slight reduction of MST2 protein levels in untreated cells. The results were quantitated from three independent experiments, and S.D. is shown. C, depletion of RASSF1A leads to reduction of phospho-MST. A 60% reduction of the RASSF1A protein level (A) is associated with an ~30% reduction of phospho-MST. The results were quantitated from three independent experiments, and S.D. is shown. D, effect of RASSF1A depletion on levels and phosphorylation status of JNK, p38, ERK1/2, and AKT in untreated and STS-treated HeLa cells. IB, immunoblot; β-tub, β-tubulin.
findings suggest that PP2A is a major phosphatase that dephosphorylates MST1/2 in vivo. This conclusion is in complete agreement with a new report published while this manuscript was in revision that shows that in Drosophila PP2A is the phosphatase that dephosphorylates the MST ortholog, the Hippo kinase (35). The situation is most likely complex and may involve feedback loops because recent results suggest that an intact MST2 signaling pathway is necessary for maintenance of PP2Ac levels in the cell (36).

The observation that MST1/2 dephosphorylation was prevented by RASSF1A suggests that RASSF1A promotes the formation of active MST1/2 by counteracting the role of phosphatases. This effect of RASSF1A on MST phosphorylation should be specific to full-length MST1/2 because the caspase-cleaved truncated forms of the kinases do not contain the C-terminal SARAH domains required for RASSF1A binding. Interestingly, it was reported that the truncated form of MST2 is remarkably resistant to dephosphorylation (23), suggesting that other RASSF1A-independent events must be responsible for irreversible autophosphorylation of truncated MST2.

Other members of the RASSF protein family (37) may have different effects on MST phosphorylation and activation. The nearest homolog of RASSF1A in Drosophila is RASSF (38). However, Drosophila RASSF is more closely related to mammalian RASSF2, RASSF4, and RASSF6. Because Drosophila RASSF1 and mammalian RASSF6 function as inhibitors of MST/Hippo kinases (38, 39), it will be interesting to test their effects on MST dephosphorylation. Slightly lower levels of MST2 were detected in RASSF1A-depleted cells (Fig. 3). Thus, loss of RASSF1A may sensitize MST2 to degradation. The RASSF family member RASSF2 has recently been shown to stabilize and protect MST2 from degradation (40). Thus, it is conceivable that some functional redundancy between RASSF1A and other RASSF proteins exists, and their relative effects on MST activity may be cell type-dependent.
We tested if the binding between MST1/2 and RASSF1A changes during induction and progression of apoptosis. The RASSF1A-MST interaction was always observed, even before treatment (Fig. 4 and supplemental Fig. S3). RASSF1A promoted the phosphorylated state of MST1/2 at early time points of apoptosis, and the emergence of apoptotic cells beginning 2 h after treatment with STS was diminished in the absence of RASSF1A (Fig. 4). At the later time points, STS treatment resulted in MST1/2 cleavage and partial RASSF1A degradation. However, a particularly stable RASSF1A-MST complex persisted. Because RASSF1A-MST complexes preexist in cells before induction of apoptosis, it is reasonable to propose that the function of RASSF1A is to provide a pool of phosphorytase-resistant MST molecules in a “poised” state. However, it still remains to be determined how autophosphorylation and initial activation of MST are accomplished upon a stimulus. Physiological inputs upstream of MST kinases other than apoptotic inducers may be important for MST signaling but remain to be identified. For example, in contrast to findings from Drosophila, no evidence was found that mammalian merlin positively regulates mammalian MST2 (36). Of note, there are several reports that have linked the function of MST kinases to centrosome duplication (41), mitotic control (7, 42, 43), and lymphocyte trafficking (44).

In summary, our data show that the role of RASSF1A in MST kinase pathways is to provide and preserve the phosphorylated/activated state of MST1 and MST2 by preventing dephosphorylation of these kinases by the protein phosphatase PP2A. This is one of the first examples of a tumor suppressor acting as an inhibitor of a specific dephosphorylation reaction.

REFERENCES

The Tumor Suppressor RASSF1A Prevents Dephosphorylation of the Mammalian STE20-like Kinases MST1 and MST2
Cai Guo, Xiaoying Zhang and Gerd P. Pfeifer

doi: 10.1074/jbc.M110.178210 originally published online January 3, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.178210

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/01/03/M110.178210.DC1

This article cites 44 references, 18 of which can be accessed free at
http://www.jbc.org/content/286/8/6253.full.html#ref-list-1