Siah1 Interacts with the Scaffold Protein POSH to Promote JNK Activation and Apoptosis

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Siah proteins are ubiquitin-protein isopeptide ligases (E3) that have been implicated in a variety of cellular actions, including promotion of apoptotic death. Here, we show that Siah1 is a binding partner for POSH (plenty of SH3s), a scaffold component of the apoptotic JNK pathway, and that Siah contributes to death of neurons and other cell types by activating the JNK pathway. Such pro-apoptotic activity requires the E3 ligase activity of Siah1. Moreover, apoptotic stimuli markedly elevate cellular Siah1 levels by a mechanism reliant on Siah1 protein stabilization. This stabilization requires JNK pathway activation and interaction with POSH and is enhanced by phosphorylation of SIAH1 at tyrosines 100 and 126. Depletion of intracellular Siah proteins via small interference RNA partially protects cells from death evoked by apoptotic stimuli such as trophic factor deprivation and DNA damage. These findings thus reveal a “loop” mechanism in which the JNK pathway promotes SIAH1 stabilization and in which SIAH1 in turn activates the JNK pathway and, ultimately, contributes to cell death.

Expression of Siah1 proteins is elevated in dying cells (3, 16), and Siah1 overexpression induces apoptotic death of U937 cells (14). In another study, however, Siah1 overexpression alone was insufficient to induce death and did so only when co-expressed with another p53-regulated gene, Pw1/Peg3 (13). Thus, the extent to which Siah1 participates in cell death and the mechanism by which it may do so remain to be more clearly defined.

The c-Jun N-terminal kinases (JNKs) play key roles in responses to cellular stress and in many paradigms of mammalian apoptotic cell death and therefore have the potential to be involved in Siah-associated apoptosis (20). A sequential apoptotic signaling cascade that leads to JNK activation has been described in neurons and other cell types (20–24) in which GTP-bound forms of Rac1 and Cdc42 lead to activation of the mixed lineage kinases (MLKs), which in turn phosphorylate and activate mitogen-activated protein kinase kinases MKK4 and -7, which in turn phosphorylate and activate JNKs. JNKs mediate apoptotic death by phosphorylating substrates such as c-Jun (23, 25) and Bim (26).

Recent studies implicate the multimain molecule POSH (plenty of SH3s) as an additional element in the apoptotic JNK pathway (27, 28). POSH was shown to interact directly with the GTP-bound, but not GDP-bound, form of Rac1 (27). In addition to a novel Rac1 binding region, POSH contains four SH3 domains and a zinc Ring finger domain (27). Expression of POSH in fibroblasts induces apoptosis (27). We further established that Posh expression stimulates the JNK pathway and induces death of neuronal and other cell types and that it participates as a required element in death of neurons evoked by withdrawal of trophic support (28). Experiments used dominant negative MLKs and an inhibitor of MLK activity placed POSH upstream of MLKs in apoptotic signaling. POSH directly binds MLK family members and interacts with downstream pathway components, including MKK4 and -7 and JNKs (28). Thus, POSH serves as a scaffold that links activated Rac1 with components of the JNK pathway and thus forms a complex to mediate apoptotic JNK activation and c-Jun phosphorylation (28). The scaffold-like properties of POSH raised the possibility that it might interact with additional proteins that might also influence the JNK pathway and cell death.

The present work addresses the issues of how apoptotic stimuli lead to elevation of cellular Siah levels and how this in turn leads to cell death. We show that Siah1 binds to POSH and participates in activation of the apoptotic JNK pathway. Moreover, we provide evidence for a loop in which apoptotic stimuli lead to stabilization of Siah1 protein via a mechanism dependent on JNK pathway activation and on Siah tyrosine phosphorylation, thereby enhancing its capacity to contribute to JNK-mediated death. Finally, we show that endogenous Siah plays a role in several cell death paradigms.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—CEP-1347 was kindly provided by Cephalon Inc. (West Chester, PA) and applied as described previously.
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(24). Hoechst dye 33342, camptothecin, anti-human recombinant NGF, and anti-FLAG monoclonal antisera were from Sigma. Other primary immunological reagents were directed against JNK and phospho-JNK (Thr-183/Tyr-185) (Cell Signaling, Beverly, MA), His tag (Novagen, Madison, WI), HA tag (Clontech, Palo Alto, CA), Myc tag, and GFP (Santa Cruz Biotechnology, Santa Cruz, CA).

Yeast Two-hybrid Screen—The two-hybrid screen was performed with the ProQuest™ two-hybrid system, Invitrogen. Mouse Posh was cloned in-frame with the GAL4 DNA binding domain in the vector pDBLeu to create pDBLeu-Posh. Ma203 yeast cells were successively transformed with pDBLeu-Posh and mouse brain library pCP86 (Invitrogen). Clones were selected for strong interactions based on screening for expression of reporter genes His, LacZ, and URA3.

Plasmids—The following constructs were generously provided as follows: pRRK5-myc-POSISH from mouse and human RAC1 and CDC42 were from Alan Hall (University College London, United Kingdom), pCDNA3.FLAG-SIAH1, pCDNA3.HA-SIAH1, and pCDNA3.FLAG-SIAH1 S41/S44 were from Eric R. Fearon (University of Michigan Medical Center, Ann Arbor, MI), and FLAG-MLK3 cDNA was from Kathleen A. Gallo (Michigan State University, East Lansing, MI), dominant negative c-jun (d/c-jun or Tam67 c-jun) was from Michael J. Birrer (National Institutes of Health, Rockville, MD).

The cDNAs for both wild-type and kinase-inactive forms of MKK4 and MKK7 in pCDNA3 and MLK1, MLK2, MLK3, DLK, and their kinase-inactive forms in pCDNA3 and pCMS-EGFP have been described previously (Xu et al. (24)), as have all Posh constructs in pCMS-EGFP and pGEX2T (Xu et al. (28)). PCMS-EGFP.FLAG-SIAH1, pCMS-EGFP.FLAG-SIAH1 S41/S44 were constructed by PCR amplification of the corresponding cDNAs in pCDNA3 (5), respectively. Primers used were the T7 promoter (bases 864–882) and the Sp6 promoter (bases 999–1016) in pCDNA3. The constructs were cloned into pCR2.1.TOPO (Invitrogen), excised with EcoRI, and cloned into pCMS-EGFP vector. pEGFP.SIAH1 and pEGFP.SIAH1 S41/S44 were constructed by PCR amplification of the corresponding cDNAs in pCDNA3 with primers 5′-aagcttaaccatgagcttctcctcctgtttttctgaatagc-3′, cutting with EcoRI/HindIII and cloning into the same site of pEGFP-N1. pEGFP.POSH was constructed by PCR amplification of Posh from pCMS-EGFP.myc-POSISH (Xu et al. (28)) with primers 5′-ctgggtgaagatgcctgctctctctg-3′ and 5′-gaagttgcgaatggatcttttttt-3′, cloning into pCR2.1.TOPO, and then cutting with EcoRI/HindIII and cloning into the same site of pEGFP-N1. pEGFP.POSH was constructed by PCR amplification of Posh from pCMS-EGFP.myc-POSISH (Xu et al. (28)) with primers 5′-ctgggtgaagatgcctgctctctctg-3′ and 5′-gaagttgcgaatggatcttttttt-3′, cloning into pCR2.1.TOPO, and then cutting with Xhol/BamHI and cloning into the same site of pEGFP-N1. The Posh fragment was excised from pEGFP-POSH with Nhel/BamHI and cloned into the same site of pDSRed-i-N1 to construct pRed-POSH. SIAH1 ΔC2 was constructed as a C-terminal deletion mutant of SIAH1 lacking the last 52 amino acids, which is deficient in its NheI/BamHI and cloned into the same site of pCMS-EGFP. SIAH1 was designed and constructed by annealing oligonucleotides—5′-ctgaaagttgcgaatggatctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
that, despite comparable efficiencies of transfection (indicated by levels of co-expressed eGFP), mutant SIAH1 was much more highly expressed than the wt form (Fig. 1, A and B). This is consistent with past evidence that cellular levels of endogenous SIAH1 are very low and that this is due to automediated degradation via the proteasomal pathway (5).

POSH also possesses a zinc Ring finger domain and putative E3 ligase activity and is more stable when this domain is deleted or mutated (28). The C-terminal 52 amino acids of SIAH1 are required for dimerization and auto-destabilization (5, 29). We found that, despite its greatly enhanced stability and consequent elevated expression, SIAH1 with these residues deleted (SIAH1 ΔC2) was severely compromised for interaction with POSH (Fig. 1C). Thus, SIAH1 either has to bind to POSH via its C terminus or has to form dimers for this interaction to occur.

To further characterize the POSH-SIAH1 interaction, we tested the ability of GST-POSH fusion proteins to pull down SIAH1 prepared in an in vitro transcription-translation system. SIAH1 binds GST-POSH (amino acid 437–892) fusion protein but not GST alone or GST-POSH (amino acid 2–460) (Fig. 1D). These observations indicate that SIAH1 binds to the C-terminal half of POSH and that this binding is likely to be direct.

Finally, to evaluate whether endogenous POSH and Siah1 interact, lysates of camptothecin-treated PC12 cells were subjected to co-immunoprecipitation and Western immunoblotting with POSH and Siah1 antisera. Camptothecin treatment was used because apoptotic stimuli significantly elevate cellular levels of both POSH (40) and Siah1 (see Fig. 4A). Analysis of the immunoprecipitates revealed clear interaction of the endogenous proteins (Fig. 1E).

**SIAH1 Induces Apoptosis—** Siah1 overexpression triggers apoptosis of U937 cells (14) and cooperates with Pw1/Peg3 to promote death of L10 cells (13). We observed that transient SIAH1 overexpression also triggers death of 293T, COS-7, and U2OS cells (Fig. 2A and data not shown). Because Siah is at high levels in the developing nervous system (2, 10) and because of the role of POSH in neuronal death (28), we additionally evaluated the effect of SIAH1 on naïve and neuronally differentiated PC12 cells and cultured sympathetic neurons. SIAH1 overexpression reduced survival of neuronal PC12 cells and increased the proportion of transfected cells with apoptotic nuclei by about 3-fold (Fig. 2B and C). Similar findings were achieved with naïve PC12 cells (data not shown) and with sympathetic neurons (Fig. 2D). SIAH1-induced cell death was characterized by cell contraction, surface blebbing, and broken, shrunken, and condensed (pyknotic) nuclei, all typical of

![FIGURE 1. SIAH1 interacts with POSH both in vivo and in vitro. A and B, SIAH1 interacts with POSH in vivo. 293 cells were transfected with expression vector (pCMS.EGFP) encoding Myc-POSH or ΔRing POSH and FLAG-SIAH1C2 or SIAH1 S41/S42, alone or in combination as indicated. 20 h later, aliquots of cell lysates were analyzed by eGFP expression to assess transfection efficiencies and for expressed tagged proteins. A, the remaining portions of the lysates were immunoprecipitated with anti-FLAG, and the immunocomplexes were analyzed by SDS-PAGE/autoradiography. Positions of molecular weight markers are indicated on the left (Mr, 10^6). The arrow indicates a band that is likely to be a premature translation product of SIAH1, endogenous SIAH1 and POSH interact with each other. PC12 cells were treated with camptothecin for 6 h. Cell lysates were subjected to immunoprecipitation with anti-POSH (α-POSH), anti-Siah1 (α-Siah), or IgG antibodies as indicated. The immunocomplexes were separately probed for SIAH1 and POSH.](image-url)
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FIGURE 2. Overexpression of SIAH1 induces apoptosis. A, 293T cells were transfected with either expression vector pCMS.EGFP (panels a–c) or expression vector encoding SIAH1 (d–f). 20 h after transfection, cells were visualized by microscopy to detect cells with GFP signaling (a and b, and d and c) or stained with Hoechst dye 33342 to visualize nuclei (c and f). Replicate cultures of neuronally differentiated PC12 cells were transfected with empty expression vector pCMS.EGFP (GFP) or pCMS.EGFP.FLAG-SIAH1 (SIAH1), b, the numbers of transfected cells per culture with an eGFP signal were counted 48 h after transfection, and the percentages of surviving cells were calculated by normalizing to the numbers of eGFP-expressing cells transfected with pCMS.EGFP. Values in B and C represent means ± S.E. (n = 3). C, two days after transfection, cultures were stained with Hoechst 33342 dye, and the percentages of eGFP-positive cells with apoptotic nuclei were determined by scoring at least 100 nuclei per culture. D, rat sympathetic neurons were transfected with the above constructs and 48 h later evaluated as in B for percentage of neurons with apoptotic nuclei. Values represent means ± S.E. (n = 3).

To test this hypothesis, we transfected SIAH1 and the Ring finger mutant SIAH1 S41/S44 and SIAH1 ΔC2, a mutant that does not bind and lead to degradation of its targets, including itself (5, 29). Although the mutant SIAH1 S41S44 and SIAH1 ΔC2 proteins are much more stable than the wt protein (Fig. 1 and supplemental Fig. 1), unlike the wt protein, they did not cause changes in cell morphology or death (data not shown). Thus, SIAH1 appears to require E3 ligase activity as well as dimerization and/or POSH interaction to induce death.

SIAH1 Activates the JNK Pathway and Acts Upstream of c-Jun to Induce Death—Because Siah1 induces death in naïve and neuronally differentiated PC12 cells (Fig. 3, C and D), to confirm the requirement for JNKs in SIAH1-induced cell death, we created two hairpin constructs that produce small interfering RNAs (siRNAs) targeted against both JNK1 and JNK2 transcripts (Xu et al. (40)). Both constructs down-regulated expression of endogenous JNKs (supplemental Fig. 2B) (Xu et al. (40)). We co-transfected FLAG-SIAH1 with JNK siRNA into neuronally differentiated PC12 cells. As shown in supplemental Fig. 2A, cell death induced by SIAH1 was suppressed significantly by co-transfection of FLAG-SIAH1 with JNK siRNA compared with that in cells co-transfected with a control siRNA.

In contrast to the above findings, SIAH1-evoked death was not suppressed by d/n forms of either Rac1 or Cdc42 (data not shown). These observations are consistent with the interpretation that SIAH1, like POSH, acts at a point in the pathway downstream of Cdc42 and Rac1, but upstream of the MLKs, JNKs, and c-Jun.

Endogenous SIAH1 Levels Are Elevated by an Apoptotic Stimulus via Activation of the JNK Pathway—Because Siah1 levels appear to be elevated in apoptotic cells and tissues (3, 16), we next determined whether endogenous SIAH1 levels were regulated by an apoptotic stimulus that activates the JNK pathway. U2OS cells were treated for 7 h with camptothecin, a topoisoamerase inhibitor that activates JNKs and evokes death of various cell types, including U2OS cells (data not shown). This treatment significantly elevated the levels of endogenous SIAH1 (Fig. 4A). Moreover, this effect was blocked by the MLK family inhibitor CEP-1347 and by SP600125, a JNK inhibitor (32), whereas the transcription inhibitor actinomycin D only partially diminished the increase (Fig. 4A). Taken together, these findings indicate that an apoptotic stimulus elevates endogenous SIAH1 protein, that this effect requires activation of the JNK pathway, and that it appears to be due, at least in part, to stabilization rather than increased synthesis of SIAH1 protein.

Regulation of SIAH1 Levels by JNK Pathway Components—Next we explored in further detail the role of JNK pathway components in SIAH1 stabilization. Co-expression of SIAH1 with POSH led to a very large increase in expression of SIAH1 protein compared with transfection with SIAH1 alone (Fig. 4B). This did not require that POSH possess its E3 ligase activity, because similar effects were achieved with ΔRing POSH (Fig. 4B, left panel). In these experiments, exogenous SIAH1 expression was driven by the CMV promoter, and so, as at least in part as with the endogenous protein, elevation of SIAH1 appeared to occur via a post-transcriptional mechanism involving protein stabilization.

Because POSH is a scaffold for the JNK pathway and SIAH1 is in the same complex, we next tested whether other pathway components also contribute to SIAH1 stabilization. Co-expression with the MLK family members MLK3 (Fig. 4, C and F, and supplemental Fig. 3), MLK2 (Fig. 4, D and E), MLK1 (Fig. 4G), or DLK (data not shown) greatly stabilized SIAH1. This effect required MLK activity, because it was repressed by the MLK inhibitor, CEP-1347 (Fig. 4, C and D) and was not promoted by catalytically inactive d/n forms of MLK2 or MLK3 (Fig. 4, D and E, and data not shown). The stabilizing action of POSH, which activates MLKs (28), was also substantially blocked by CEP-1347 (Fig. 4B). Because transfected POSH and MLKs activate endogenous JNKs, we next asked whether SIAH1 stabilization requires JNK activation. As shown in Fig. 4F, co-transfection of SIAH1 and MLK3 with a d/n MKK7 construct, which acts downstream of MLKs in the pathway and interferes with JNK activation (24, 33), suppressed SIAH1 stabilization. A similar effect was achieved with the JNK inhibitor, SP600125 (Fig. 4F). To confirm the SP600125 result, SIAH1 was co-transfected with MLK1 either in the presence or absence of d/n JNK1. d/n JNK1 almost totally abolished MLK1-induced stabilization of SIAH1 (Fig. 4G).
FIGURE 3. SIAH1 induces phosphorylation of JNKs and induces neuronal death through the JNK pathway. 293 cells were transfected with pCMc-EGFP or with SIAH1, SIAH1 S41/S44, or POSH in pCMc-EGFP as indicated. CEP-1347 was added as indicated 4 h after transfection at a final concentration of 200 nM. Cell lysates were analyzed for the levels of phospho-JNK by Western blotting. The blot was re-probed with JNK as a loading control and then with eGFP antibody as a transfection control. B, death induced by SIAH1 expression is suppressed by the JNK pathway inhibitor, CEP-1347. pCMc-EGFP and SIAH1 in pCMc-EGFP vector were co-transfected into neuronal PC12 cells either with pRK5 or with pRK5.myc-POSH (POSH in figure). Where indicated, cultures were pretreated and maintained with 200 nM CEP-1347. Two days after transfection, numbers of surviving transfected cells were assessed by strip counting. Values represent average number of surviving transfected neurons per strip and are means from three cultures ± S.E. C and D, apoptotic death induced by SIAH1 is suppressed by d/n c-Jun. pCMc-EGFP or SIAH1 in pCMc-EGFP were co-transfected with either pCMc vector or pCMV d/n c-Jun into neuronal PC12 cells. Two days after transfection, numbers of surviving transfected cells (C) and the percentages of apoptotic nuclei (D) were assessed as in B and Fig. 3, respectively. Values in A, C, and D are the means of three cultures ± S.E. Similar results were obtained in two additional independent experiments.

FIGURE 4. Siah1 levels increase in response to DNA damage and to activators of the JNK pathway. A, camptothecin elevates endogenous Siah1 by a mechanism requiring JNK pathway activity and that is at least partially independent of transcription. U2OS cells were treated with 10 μM camptothecin, 0.1 μg/ml CEP-1347, or SP600125 (20 nM), JNK inhibitor (SP600125, 20 μM), or actinomycin D (0.1 μg/ml). Cell lysates were subjected to Western immunoblotting with anti-Siah1, and the blot was re-probed with anti-ERK to verify equal loading. B-F, regulation of SIAH1 expression by the JNK pathway. pCMS.EGFP.FLAG-SIAH1 was co-transfected into 293 cells either with empty vectors pCMS.EGFP (all cDNA constructs in Fig. 5 (B–D) are in pCMS.EGFP) or pCDNA3 (in Fig. 5 (E and F), MLK3, d/n MKK7, and d/n c-Jun are in pCDNA3 or with wt or mutant forms of the indicated JNK pathway components. Numbers above blots indicate micrograms of each construct used for co-transfection. SP600125 (20 μM) and CEP-1347 (200 nM) were present where indicated. Cell lysates were analyzed by immunoblotting for the levels of transfected gene products (anti-FLAG antiserum for SIAH1) and for phospho-JNKs as indicated. Blots were re-probed with anti-ERK to verify equal loading. B, POSH promotes the stabilization of SIAH1 by a mechanism independent of the Ring finger of POSH. C–E, MLK3 and MLK2, but not d/n MLK2 induce the stabilization and decreased electrophoretic mobility of SIAH1 by a CEP-1347-sensitive mechanism. In panel D, the amount of protein loaded in lane 6 is 1/10 that of that in lane 2 to facilitate detection of the shift in electrophoretic mobility. In panel E, the ratio of HA-d/n MLK2 to HA-MLK2 plasmid used for transfection was increased by 3-fold to yield a similar final expression level of each protein product. F, stabilization of SIAH1 by MLK3 co-expression is suppressed by d/n MKK7 and SP600125. G, stabilization of SIAH1 by MLK1 co-expression is suppressed by d/n JNK1.
To more fully evaluate the role of JNKs in stabilization of SIAH1 induced by MLKs, we transfected 293 cells with the JNK siRNAs used above (supplemental Fig. 2A), and 48 h later, FLAG-SIAH1 was co-transfected with FLAG-MLK3. As shown in supplemental Fig. 2B, co-transfection of FLAG-SIAH1 with FLAG-MLK1 and JNK siRNA led to a significant fall of FLAG-SIAH1 expression compared with that in cells co-transfected with a control siRNA. That only partial suppression of SIAH1 expression was induced by JNK siRNAs in this experiment most likely reflects the fact that only a portion of the cell population was transfected on each occasion (~80% in this experiment).

Taken together, the above observations indicate not only that SIAH1 activates the JNK pathway, but also that JNK pathway activation in turn stabilizes SIAH1. To test whether endogenous POSH plays a required role in SIAH1 stabilization, we next generated cell lines (293 siPOS embryonic stem cells) in which POSH expression is knocked-down by constitutive expression of POSH siRNA (Fig. 5A). Camptothecin treatment for 8 h elevated the levels of endogenous SIAH1 in wt 293 cells, but not in 293 siPOS cells (Fig. 5B). Additionally, SIAH1 and MLK1 were co-transfected into both 293 and 293 siPOS cells and assessed for SIAH1 expression (Fig. 5C). Despite equal levels of transfection (as shown by eGFP expression), both the basal and MLK1-stimulated levels of SIAH1 expression were substantially reduced in the 293 siPOS cells. Similar results were achieved with MLK3 and with independent lines of 293 siPOS cells (supplemental Fig. 3). Thus, POSH plays an essential role in stabilization of endogenous and exogenous SIAH1.

Because POSH is essential for SIAH1 stabilization, we next investigated whether POSH plays a role in cell death induced by SIAH1 overexpression. SIAH1 was transfected into either 293 or 293 siPOS cells. Death induced by SIAH1 in the siPOS cells was significantly (by ~30%) reduced as compared with that in wt cells, indicating that endogenous POSH does play a role in this process. The observation of only partial protection from overexpressed SIAH1 in the siPOS cells could reflect the activation of a POSH-independent mechanism when high enough expression of SIAH1 is achieved.

Because SIAH1 is responsible for its own ubiquitination and degradation, it was possible that activation of the JNK pathway promotes stabilization of SIAH1 by interfering with its self-directed E3 ligase activity. To test this possibility, tagged SIAH1, SIAH1 ΔC2, or SIAH1 S41/44 in pCMS.EGFP were transfected either alone or together with MLK2 into 293 cells. The expressed SIAH proteins were then immunoprecipitated and probed on Western immunoblots with the appropriate anti-tagging antibodies and, after stripping, with anti-ubiquitin antisera. Immunoprecipitates from cells transfected with SIAH1 alone showed little detectable SIAH protein or, consequently, of ubiquitinated SIAH1 (supplemental Fig. 4A). However, in replicate cultures treated with the proteasome inhibitor MG132 the immunoprecipitate showed both detectable SIAH expression and a number of higher weight ubiquitinated species (supplemental Fig. 4B). Analysis of immunoprecipitates from cells co-transfected with SIAH1 and MLK2 revealed not only SIAH1 protein, but also the presence of a ladder of ubiquitinated slower mobility species (supplemental Fig. 4, A and B). In contrast, and as anticipated, immunoprecipitates from cells transfected with activity-deficient SIAH1 ΔC2, or SIAH1S41/544, either alone or together with MLK2, did not show the presence of any detectable ubiquitinated species (supplemental Fig. 4, A and B). Taken together, these observations indicate that, even after activation of the JNK pathway, SIAH retains E3 ligase activity, at least toward itself.

Tyrosine Phosphorylation Mediates Stabilization of SIAH1 by the JNK Pathway and Enhances Interaction of SIAH1 with POSH—We noted that in some experiments SIAH1 stabilization correlated with a small decrease in its electrophoretic mobility (Fig. 4D). This led us to examine whether such stabilization might be mediated by phosphorylation. We first used metabolic labeling with [32P]orthophosphate followed by immunoprecipitation to confirm that SIAH1 is phosphorylated when co-transfected with MLK1 (Fig. 6A). We next evaluated whether the phosphorylation changes induced by apoptotic stimuli include Tyr residues and whether this occurs for endogenous SIAH1. Lysates of PC12 cells treated with camptothecin or Me2SO control were subjected to immunoprecipitation with anti-SIAH1 antisera and to Western blotting with anti-P-Tyr antibody (Fig. 6B). Camptothecin treatment elevated both the expression and Tyr phosphorylation of SIAH1.

Next, to assess the site(s) of SIAH1 tyrosine phosphorylation, whether such phosphorylation is subject to regulation by JNK pathway stimulation, and whether it plays a role in SIAH1 stabilization, we separately (and in some cases, in combination) mutated the five Tyr residues in SIAH1 to Phe and co-expressed the mutants and wt proteins with or without His-MLK1. Mutation of Y100F and Y126F each reduced MLK1-stimulated SIAH1 Tyr phosphorylation, whereas mutation of the other three sites almost fully abolished such phosphorylation (Fig. 6C, left panel). In contrast, mutation of the other three sites (Y47F, Y199F, and...
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Y223F) had no apparent effect on the level of SIAH Tyr phosphorylation (data not shown). When we evaluated stabilization of the mutants by co-expressed MLK1, SIAH1 Y100F and Y126F were each only partially stabilized compared with wt SIAH1, whereas stabilization of the Y100F/ Y126F double mutant was even less apparent (Fig. 6C, right panel). These two Tyr residues are in the substrate binding domain of SIAH1. Mutation of Tyr-47, Tyr-199, or Tyr-223 had no apparent effect on the level of SIAH Tyr phosphorylation (data not shown). Taken together, these observations indicate that activation of the JNK pathway leads to phosphorylation of SIAH1 on Tyr-100 and Tyr-126 and that this in turn enhances its interaction with POSH and consequent stabilization.

**Endogenous Siah Proteins Contribute to Cell Death**—To assess the potential functional role of endogenous Siah in cell death, we created several hairpin loop constructs that produce siRNAs targeted against Siah transcripts. Construct pSuppressor.siSiah1 was designed to knock down levels of all Siah family proteins including Siah1a, Siah1b, and SIAH1 proteins, whereas pSuppressor.siSiah1&2 was designed to knock down the levels of all Siah proteins, including Siah1a, Siah1b, Siah2, SIAH1, and SIAH2. As a control, pSuppressor.siSiah1m was designed in the same way as pSuppressor.siSiah1 except that two point mutations were introduced into the siRNA hairpin region. To confirm the efficacy of the constructs, they were co-transfected into 293 cells together with pCMS-EGFP.FLAG-SIAH1 S41S42 (Fig. 7A) or pCMS-EGFP.FLAG-SIAH1 (supplemental Fig. 5), and the levels of both endogenous SIAH1 and the FLAG-tagged proteins were assessed 2 days later. Both constructs effectively diminished expression of both endogenous and exogenous SIAH1 (Fig. 7A and supplemental Fig. 5). However, the pSuppressor.siSiah1 construct was somewhat less effective than pSuppressor.siSiah1&2 in down-regulating overexpressed FLAG-tagged SIAH1 or SIAH1 S41S42. The emporor vector and pSuppressor.siSiah1m had no apparent effect on SIAH1 expression.

We first tested the Siah siRNAs in a neuronal PC12 cell DNA damage model in which death can be suppressed by blocking the JNK pathway (31). Both siRNA constructs conferred significant protection from camptothecin (Fig. 7B). The pSuppressor.siSiah1&2 construct was consistently somewhat more effective, although it is unclear if this was due to its broader scope of action or to its greater efficacy. pSuppressor.siSiah1 also showed protection of 293 cells from camptothecin-induced death as compared with control pSuppressor.siSiah1m (Fig. 7C). In addition, both pSuppressor.siSiah1 and pSuppressor.siSiah1&2 but not the control siRNA construct completely suppressed the camptothecin-induced elevation of endogenous SIAH1 levels (Fig. 7D). NF2 deprivation is an additional model in which death requires JNK activation and phosphorylation of c-Jun (21, 23, 24, 31). Death of neuronal PC12 cells and cultured sympathetic neurons triggered by NF2 deprivation was significantly decreased by the pSuppressor.siSiah1&2 siRNA construct.

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**FIGURE 6.** MLK1 induces SIAH1 stabilization through Tyr phosphorylation and the interaction between SIAH1 and POSH. A, MLK1 induces phosphorylation of SIAH1. FLAG-SIAH1 in pCM-S. EGFP was transfected/co-transfected with pCDNA3.MLK1 into 293T cells. 18 h later, cells labeled with [32P]orthophosphate, and cell lysates were subjected to immunoprecipitation. The immunocomplexes were visualized by SDS-PAGE followed by autoradiography. B, camptothecin induces the Tyr phosphorylation of endogenous Siah1. PC12 cells were treated with camptothecin for 6 h and cell lysates (~10 mg of protein) were subjected to immuno precipitation with anti-Siah1 (aSiah1) antiserum as indicated. The immunocomplexes were probed with anti-P-Tyr and anti-Siah1 antiserum. C, MLK1 induces SIAH1 stabilization through Tyr phosphorylation. FLAG-SIAH1 and different SIAH1 Tyr to Phe point mutations (Y100F, Y126F, and Y100F/Y126F; 3 μg each) in pCM-S. EGFP were co-transfected with pCDNA3.MLK1 (1 μg) into 293T cells. Cell lysates were analyzed 15 h later by Western immunoblotting with anti-P-Tyr and/or anti-FLAG. The blot was re-probed with eGFP antibody as a transfection control (right panel). D, Tyr phosphorylation enhances the interaction between SIAH1 and POSH. 293 cells were transfected with an expression vector (pCMS. EGFP) encoding FLAG-SIAH1 and Myc-ΔRing POSH in pRK5 with or without pCDNA3 encoding His-MLK1, as indicated. 17 h later, cell lysates were analyzed for the expression of tagged proteins (left panel). The remaining portions of the lysates were immunoprecipitated with anti-Myc antibody, and the immunocomplexes were analyzed for SIAH1 with anti-FLAG antibody. The membrane was re-probed with anti-Myc antiserum to detect ΔRing POSH.

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2 Z. Xu, and L. A. Greene, unpublished findings.
SIAH1 Promotes JNK Activation and Apoptosis

DISCUSSION

Siah and Cell Death—The present study addresses several issues regarding the role of Siah proteins in cell death. First, to what degree does Siah contribute to cell death? Second, by what mechanism might it do so in addition to those already described? Third, how are Siah levels elevated in cells in response to apoptotic stimuli? Overall, our data support a loop mechanism for the participation of Siah in cell death that can be summarized as follows: 1) Siah turns over rapidly in healthy cells, resulting in a low level of expression. 2) Apoptotic stimuli lead to elevation of cellular Siah levels by protein stabilization. This stabilization requires interaction with POSH and JNK pathway activity and is enhanced by phosphorylation of Siah1 on tyrosine residues 100 and 126. 3) Elevated Siah levels promote further activation of the JNK pathway, thereby contributing to apoptotic cell death. In the discussion to follow, we further elaborate on this model and the data that support it.

SIAH1 Associates with POSH, Induces Apoptosis by Activating the JNK Pathway, and Plays a Functional Role in Cell Death—The major contribution of JNK activation to cell death in a variety of apoptotic paradigms has underscored the importance of identifying the molecules that contribute to this event. Past work identified POSH as a proximal element of the JNK pathway that acts as a scaffold for MLKs and other

FIGURE 7. Siah siRNAs protect cells from apoptotic stimuli. A, Siah siRNAs block expression of exogenous FLAG-SIAH1 S41S44. pSuppressor, pSuppressor.ssiSiah1, pSuppressor.ssiSiah1m, or pSuppressor.ssiSiah1&2 (labeled as Vect, ssiSiah1, ssiSiah1m, and ssiSiah1&2, respectively) were co-transfected with pCMS.EGFP-FLAG-SIAH1 into neuronal PC12 cells. 36 h later, the cultures were monitored by Western immunoblotting for expression of FLAG-SIAH1 S41S44 with Siah1 antiserum. The blot was re-probed with anti-GFP to indicate transfection efficiencies. B–F, Siah1 siRNAs promote neurally differentiated PC12 cells from camptothecin-induced death. pSuppressor, pSuppressor.ssiSiah1, or pSuppressor.ssiSiah1&2 were co-transfected with pCMS.EGFP into neurally differentiated PC12 cells. Two days after transfection, cells were treated with camptothecin (10 μM). Numbers of transfected cells in each culture were assessed 0, 24, and 48 h after camptothecin treatment. The percentages of surviving transfected cells at 24 and 48 h of exposure are expressed relative to the numbers of transfected cells present in the same cultures 0 h after treatment. Values represent mean ± S.E. for three replicate cultures. Difference from control: *, p < 0.001; **, p < 0.002. G, Siah siRNA protects cultured sympathetic neurons from NGF deprivation. pSuppressor or pSuppressor.ssiSiah1 were co-transfected with pCMS.EGFP into sympathetic neurons and the percentages of surviving cells following 24 h of deprivation are expressed relative to the numbers of transfected cells without treatment. Values represent mean ± S.E. for three replicate cultures. Difference from control: *, p < 0.002; **, p < 0.001; ***, p < 0.002. Two days after transfection, cells were treated with NGF antibody for 5 h. Cells were then fixed and stained for the expression of phosphorylated c-Jun (P-c-Jun) with P-c-Jun antibody. Values represent mean ± S.E. for three replicate cultures. Difference from control siRNA: *, p < 0.008.
pathway components upstream of JNKs (24). Here we found that POSH also directly interacts with Siah proteins, and this in turn led us to demonstrate that SIAH1 has a role in regulating the JNK pathway, thereby promoting apoptosis.

Our data indicate that SIAH1 interacts with the POSH C-terminal domain. Consistent with this, we noted that there are three potential binding motifs for SIAH1 in this region. One of them, RPTAAVTP, is conserved from rodent to human and matches the core sequence, PXAXYXP, found in several SIAH-interacting proteins (34).

Prior studies have indicated links between Siah proteins and cell death (3, 13, 16). However, the role of endogenous Siah in cell death has not been clear, and there has been only limited information about the mechanisms by which Siah evokes apoptosis. Hara et al. (35) recently reported that SIAH1 plays a role in nitric oxide-dependent cell death by promoting nuclear translocation of S-nitrosylated glyceraldehyde-3-phosphate dehydrogenase. Our findings establish, in a set of different apoptotic paradigms that do not involve NO formation, that SIAH1 (and probably other family members) activates the JNK pathway and promotes death through this mechanism. Moreover, our results implicate endogenous Siah proteins in the apoptotic mechanism. Thus, reduction of Siah expression via siRNA constructs suppressed cell death in several different apoptotic paradigms. Given the widespread expression of Siah genes (2) and the involvement of the JNK pathway in a wide variety of apoptotic models (25, 36), it seems likely that Siah family members will contribute to cell death under a variety of circumstances.

Although interference with Siah expression conferred protection from apoptotic stimuli, we did not observe total rescue in our studies. This contrasts with effects of other agents that interfere with the JNK pathway and that provide near complete protection (24). One possibility is that we did not fully suppress expression of all Siah family members in our siRNA experiments. However, this seems unlikely given the design of our siRNA to affect all family members and its efficacy on expression of SIAH1. An alternative explanation is that, although Siah significantly contributes to the apoptotic mechanism, it is not absolutely indispensable for death.

Apoptotic Stimuli Elevate SIAH1 Levels by a Non-transcriptional Mechanism—Observations, by ourselves and others, that Siah overexpression leads to apoptotic death indicate that the level of Siah proteins must be kept at low levels in un-stressed cells to assure their survival. This is consistent with the difficulty in detecting Siah proteins in most viable cells. The apoptotic action of overexpressed Siah also suggests a context for the findings that endogenous SIAH1 levels greatly increase in response to an apoptotic stimulus (present work) and that Siah expression is elevated in dying cells (3, 16). Such increases in endogenous Siah proteins would reach a threshold sufficient to contribute to activation of the JNK pathway and to cell death.

Siah transcription is p53-inducible (12, 14) and was proposed to play a role in p53-mediated cell death and/or cell-cycle arrest (13, 14), although recent findings show normal p53-dependent cell-cycle arrest by cells lacking expression of Siah proteins (37). In our study, there was a substantial camptothecin-induced elevation of SIAH1 even when transcription was blocked. Because the transcription inhibitor was present for 1 h before and during the 7 h of the experiment, it is possible that the apparent partial block of induction was due to turnover of the low basal levels of the protein and that an even greater proportion of the response is non-transcriptional. The presence of a non-transcriptional mechanism for Siah regulation is further supported by our observation that pro-apoptotic activators of the JNK pathway markedly enhanced the expression of exogenous SIAH1 driven by the CMV promoter.

The most likely mechanism for non-transcriptional elevation of SIAH1 in stressed cells is by protein stabilization. SIAH1 possesses E3 ligase activity and forms homodimers that signal its own proteasomal degradation. As shown elsewhere (5) and in the present work, a SIAH1 mutant with compromised E3 ligase activity is much more highly expressed than the wt protein. We also observed higher levels of SIAH1 expression in the presence of proteasome inhibitors, providing further support for the role of degradation in regulating cellular levels of Siah proteins. This finding also may be relevant to reports that proteasomal inhibitors promote JNK activation as well as cell death and that proteasomal dysfunction and activation of the JNK pathway may contribute to the pathophysiology of disorders such as Parkinson and Alzheimer diseases (38, 39).

A Feed-forward Loop Mechanism for SIAH1 Stabilization by POSH and MLKs—We found that elevation of endogenous SIAH1 by camptothecin was entirely blocked by inhibitors of the JNK pathway. Moreover, expression of exogenous SIAH1 was greatly increased by co-expression with JNK pathway activators, and this effect was suppressed by JNK pathway inhibition. This suggests a feed-forward loop mechanism in which initial activation of the JNK pathway leads to stabilization of SIAH1 and to an increase in its intracellular levels. In turn, elevated SIAH1 then promotes further activation of the JNK pathway, thereby contributing to death. We recently found that apoptotic stimuli also lead to stabilization of POSH by a mechanism dependent on JNK activation (Xu et al. (40)). Thus, elevated levels of both POSH and Siah1 may cooperate to promote cell death.

Our findings indicate that interaction with POSH is a key component of the mechanism by which SIAH1 is stabilized by apoptotic stimuli. Thus, SIAH1 was not stabilized by camptothecin or MLKs in cells with abrogated POSH expression, and SIAH1 mutants defective for interaction with POSH were less subject to MLK-mediated stabilization. There are several mechanisms by which POSH may induce SIAH1 stabilization. The C-terminal 52 amino acids are required for SIAH1 dimerization and E3 ligase activity as well as interaction with POSH. It is therefore conceivable that POSH competitively blocks SIAH1 dimerization and E3 ligase activity. However, an in vitro competition assay does not support this model (data not shown). An alternative and more likely mechanism is that SIAH1 associates with POSH as a dimer but, when complexed with POSH, is no longer subject to degradation and therefore accumulates.

We found that SIAH1 association with, and stabilization by, POSH are regulated by phosphorylation of tyrosines 100 and 126. This phosphorylation appears to be driven by activation of the JNK pathway. However, it remains to be determined whether SIAH is directly phosphorylated by pathway members or is a target for kinases that are themselves regulated by the pathway. Tyr phosphorylation does not appear to be a requisite for association of SIAH1 with POSH, because the two form complexes in yeast and in an in vitro assay. However, our findings indicate that phosphorylation of SIAH at tyrosines 100 and 126 substantially elevates the efficiency of this interaction. Taken together, our observations further extend the feed-forward loop model described above. Initial activation of the JNK pathway by apoptotic stimuli leads to Tyr phosphorylation of SIAH1. This favors association with POSH and consequent stabilization of SIAH by impairing its proteasomal destruction. Elevated SIAH1 levels then contribute to further activation of the JNK pathway and so forth until a level of pathway activation is reached that is sufficient to trigger death.

E3 Ligase Activity Is Essential for SIAH1-evoked Death—The mechanism by which SIAH contributes to activation of the apoptotic JNK pathway is presently unclear. We show here that, in contrast with wt
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SIAH1, a Ring finger mutant (SIAH1 S41/S44) with compromised E3 ligase activity and SIAH1 ΔC2, which fails to target and degrades its substrate, are unable to induce cell death. In accordance with this, although MLKs induce the stabilization of SIAH1 substantially, they do not block the E3 ligase activity of SIAH1, at least toward SIAH1 itself. In further support of this notion, expression of wt SIAH1 but not SIAH1 S41/S44 activated the JNK pathway. Such findings indicate that the pro-apoptotic activity of SIAH1 is linked to its capacity to target proteins for proteasomal destruction. This raises the attractive possibility that association of SIAH with POSH and its intracellular accumulation permit it to promote degradation of molecules that otherwise function to suppress activation of the JNK pathway. Identification of such targets is currently underway.

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