Regulation of Apoptotic c-Jun N-Terminal Kinase Signaling by a Stabilization-Based Feed-Forward Loop†

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A sequential kinase cascade culminating in activation of c-Jun N-terminal kinases (JNKs) plays a fundamental role in promoting apoptotic death in many cellular contexts. The mechanisms by which this pathway is engaged in response to apoptotic stimuli and suppressed in viable cells are largely unknown. Here, we show that apoptotic stimuli increase endogenous cellular levels of pathway components, including POSH, mixed lineage kinases (MLKs), and JNK interacting protein 1, and that this effect occurs through protein stabilization and requires the presence of POSH as well as activation of MLKs and JNKs. Our findings suggest a self-amplifying, feed-forward loop mechanism by which apoptotic stimuli promote the stabilization of JNK pathway components, thereby contributing to cell death.

Activated c-Jun N-terminal kinases (JNKs) play major roles in a variety of mammalian apoptotic cell death paradigms (6, 12, 15, 17, 22, 26, 28, 30, 31). One sequential pathway by which apoptotic stimuli promote JNK activation commences with formation of the GTP-bound forms of Rac1 and Cdc42. These small G proteins promote autophosphorylation and activation of the mixed lineage kinases (MLKs), which in turn phosphorylate and activate mitogen-activated protein kinase kinases 4 and 7 (MKK4/7), which phosphorylate and activate JNKs. Several scaffold proteins also contribute to activation of this pathway. Of these, plenty of SH3's (POSH) is a multidomain protein that binds both GTP-Rac1 and MLKs and that promotes activation of the latter (29). JNK interacting proteins (JIPs) also interact with MLKs as well as MKK4/7 and JNKs and facilitate JNK activation (27, 28, 32). Interference with the expression or activity of any of these components protects cells from apoptotic stimuli (28–30).

An important unsettled question about this apoptotic signaling pathway (referred to hereafter as the JNK pathway) concerns the means by which it is suppressed in viable cells. Overexpression of wild-type (wt) POSH, MLKs, or MKK4/7 leads to JNK activation and cell death (24, 29, 30), indicating that their endogenous expression must be suppressed in viable cells to avoid triggering inappropriate cell death. A second and related unresolved question is how the JNK pathway becomes rapidly activated in response to apoptotic stimuli. The position of the apoptotic JNK pathway upstream of transcriptional regulation implies a mechanism that works independently of regulated synthesis.

Here, we provide evidence for a self-amplifying, feed-forward loop mechanism in which apoptotic stimuli lead to en-

hanced stability and expression of multiple JNK pathway components, including POSH, MLKs, and JIPs. These effects require MLKs as well as JNK activity and are propagated through the pathway itself. The scaffold protein POSH also plays an essential role in these events.

MATERIALS AND METHODS

Materials. CEP-1347 was kindly provided by Cephalon Inc. (West Chester, PA) and applied as described previously (30). SP600125, SB203580, and U0126 were purchased from Calbiochem (La Jolla, CA). Purified His-tagged POSH peptide (amino acids 255 to 365 from mouse) was injected into rabbit to generate POSH antiserum. Primary antisera/antibodies were obtained from commercial sources as follows: phospho-JNK (Thr183/Tyr185), MLK3, and phospho-MLK3 (New England Biolabs, Beverly, MA); His tag (Novagen, Madison, WI); hemagglutinin (HA) tag (Clontech, Palo Alto, CA); Myc tag, ERK1, JNK1/2, and green fluorescent protein (GFP) (Santa Cruz, Santa Cruz, CA); JIP1 (BD Transduction Laboratories, San Diego, CA). Anti-Flag, -HA, and -Myc agarose affinity gels were purchased from Sigma (St. Louis, MO).

Cell culture. Neuronal differentiation of PC12 cells with neuronal growth factor (NGF) and promotion of death by NGF withdrawal or camptothecin treatment were achieved as previously described (29, 30), as was the culture of cortical neurons (23). 293, HeLa, Sy5S, and U2OS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The final concentrations for treatment of cells were as follows: sorbitol, 0.4 M; CEP-1347, 200 nM; camptothecin (Sigma, St. Louis, MO), 5 or 10 μ M, as indicated in the figure legends; SP600125 (Calbiochem, San Diego, CA), 20 μ M.

cDNA constructs in mammalian expression vectors. pRK5.myc-POSH from mouse was kindly provided by Alan Hall (University College London, London, United Kingdom). Wild-type MKK7, MKK4, and MKK7 (K149A), *dln* MKK4 in pCDNA3 and MLK1, MLK2, MLK3, DLK and their kinase-inactive forms in pCDNA3 and pCMS-EGFP have been described previously (29, 30). All POSH constructs in pCMS-EGFP have been described previously (29). Myc-MKK4 and Myc-MKK7 in pCMS-EGFP were constructed by replacing the POSH fragment in pCMS-EGFP.Myc-POSH (29, 30) with MKK4 and MKK7 from pRK5.Myc-MKK4 and pRK5.Myc-MKK7 using BamHI/EcoRI.

pSIREN-RetroQ-dsRed.siJNK-A and pSIREN-RetroQ-dsRed.siJNK-B were designed by selecting two target sequences which are shared by different isoforms of both JNK1 and JNK2, GATCATGAAAGAATGTCCTACC and CATGAAAGAATGTCCTACCTTC. They were converted to two pairs of complementary oligonucleotides encoding a hairpin structure by using software provided at the BD Biosciences website. Oligonucleotides were annealed separately and cloned into the BamHI/EcoRI site of pSIREN-RetroQ-dsRed (BD Biosciences, Palo Alto, CA). pSIREN-RetroQ-dsRed.siLuc was also constructed according to the manufacturer's protocol.

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FIG. 1. Apoptotic stimuli elevate the levels of endogenous MLK3 by a mechanism requiring protein stabilization and activation of MLKs and JNKs. A. Camptothecin elevates both expression and phosphorylation/activation of endogenous MLK3. HeLa cells were treated with 10 μ M camptothecin for the indicated times. Cell lysates were analyzed with MLK3 antiserum for total MLK3 expression (upper panel) or with P-MLK3 (pT277/pS281) antiserum for phospho-MLK3 (lower panel) by Western blotting. The blot was stripped and reprobed with MLK3 antiserum for total MLK3 expression (lower panel) and reprobed with JNK1 or ERK1 as loading control. B. Sorbitol elevates expression and phosphorylation/activation of endogenous MLK3. A431 cells were treated with 0.4 M sorbitol for 4 h. Cell lysates were analyzed for phospho-MLK3 level with P-MLK3 (pT277/pS281) antiserum by Western blotting. The blot was stripped and reprobed with MLK3 antiserum for total MLK3 expression and reprobed with ERK1 as loading control. C. NGF deprivation elevates the expression and phosphorylation/activation of endogenous MLK3. Neuronal PC12 cells were deprived of NGF for the indicated times. Cell lysates were analyzed for MLK3 with MLK3 antiserum. The blot was stripped and reprobed with JNK1 antiserum as loading control. D. Camptothecin elevates endogenous MLK3 in cultured cortical neurons. Cultured cortical neurons were treated with 10 μ M camptothecin for the indicated times. Cell lysates were analyzed for MLK3 expression as for panel C. E. Camptothecin elevates both expression and phosphorylation/activation of endogenous MLK3 and phosphorylation of JNKs in PC12 cells. Naïve PC12 cells were treated with 10 μ M camptothecin for the indicated times and analyzed as for panel A in addition to probing with P-JNK antiserum. F. CEP-1347 and SP600125, but not actinomycin D, block camptothecin-elevated expression and phosphorylation of endogenous MLK3. HeLa cells were pretreated with CEP-1347 (200 nM), SP600125 (20 μ M), or 0.1 μ g/ml actinomycin D for 2 h before treatment with 5 μ M camptothecin for 6 h. Cell lysates were analyzed for MLK3 expression with MLK3 antiserum by Western blotting. The blot was stripped and reprobed with JNK1 antiserum as loading control.

pSilencer 2.1-U6 neo.siJNKs-1 and pSilencer 2.1-U6 neo.siJNKs-2 were designed by selecting two target sequences which are shared by different isoforms of both JNK1 and JNK2, CTAGAAGAATTTCAAGATGT and ATATTGAT CAGTGAATAAAG. They were converted to two pairs of complementary oligonucleotides encoding a hairpin structure by using software provided at the Ambion website. Oligonucleotides were annealed separately and cloned into the BamHI/HindIII site of pSilencer 2.1-U6 neo (Ambion, Austin, TX).

Coimmunoprecipitation, Western immunoblotting, and immunostaining assays. Coimmunoprecipitation, Western immunoblotting, and immunostaining were performed as previously described (29, 30).

RESULTS

Apoptotic stimuli elevate endogenous MLK3 by a posttranscriptional mechanism. We first examined the effects of apoptotic stimuli on endogenous levels of the representative MLK family member, MLK3. As shown in Fig. 1A, endogenous MLK3 protein was rapidly elevated in HeLa cells treated with

the DNA-damaging agent camptothecin, a potent inducer of the JNK pathway and death in these cells (data not shown). MLK3 species of slower electrophoretic mobility were detected, suggesting MLK3 phosphorylation and activation (13, 19). Reprobing with an antibody specific for MLK3 phosphorylated at T277/S281, sites critical for MLK3 activation (14), confirmed that MLK3 was phosphorylated after camptothecin treatment (Fig. 1A).

Endogenous MLK3 was also rapidly elevated and phosphorylated/activated in additional apoptotic models, including A431 cells exposed to 0.4 M sorbitol, a potent inducer of cell stress and of death (Fig. 1B); NGF-deprived neuronally differentiated PC12 cells (Fig. 1C); and camptothecin-treated cultured cortical neurons (Fig. 1D), naïve PC12 cells (Fig. 1E), and 293 cells (see Fig. 5D, below).

To distinguish whether the elevation of MLK3 was due to

FIG. 2. Stabilization of exogenous MLKs requires MLK activity. 293 cells were transfected with different tagged wt or d/n *MLK* family members in the pCMS.EGFP vector as indicated. CEP-1347 was added as indicated 4 h after transfection to a final concentration of 200 nM. Cell lysates were analyzed for levels of MLK3 with MLK3 antibody (A), MLK2, d/n MLK2 (B), or DLK (D) with HA antibody or MLK1 with His antibody (C) by Western blotting. Where indicated, blots were reprobed with phospho-MLK3 antibody or phospho-JNK (P-JNK) antibody. Blots were also reprobed where indicated with GFP antibody as a control for transfection efficiency and with ERK1 antibody as loading control.

either transcriptional or posttranscriptional events, we co-treated HeLa cells with camptothecin and the transcriptional inhibitor actinomycin D. The inhibitor had very limited effect on elevation of MLK3 protein, thus favoring a largely post-transcriptional mechanism (Fig. 1F).

Elevation of MLKs by apoptotic stimuli requires MLK activity. To determine whether elevation of endogenous MLKs in response to apoptotic stimuli requires MLK phosphorylation/activation, we exposed HeLa cells to camptothecin and the selective MLK inhibitor CEP-1347 (18). Under these conditions, elevation of endogenous MLK3 was completely blocked (Fig. 1E).

To further explore the role of MLK activation in regulating MLK levels during apoptotic induction, we transfected 293 cells with epitope-tagged wild-type (wt) and dominant-negative (d/n) forms of *MLK3* in the pCMS.EGFP vector. The d/n *MLK3* is kinase inactive and blocks cell death promoted by apoptotic stimuli, whereas wt *MLK3* induces apoptotic death by autoactivation and stimulation of the JNK pathway (30). As shown in Fig. 2A, there was significantly higher expression of wt *MLK3* protein compared with d/n *MLK3*. Because expression of both proteins was driven by the exogenous cytomegalovirus promoter, this effect is likely due to differences in protein stabilization and not to transcriptional regulation. Similar to endogenous *MLK3* under apoptotic conditions, overexpressed *MLK3* was activated as indicated by recognition with anti-P-*MLK3* (T277/S281) antibody (Fig. 2A). These findings indicate that *MLK3* stabilization requires *MLK3* kinase activity. In further support, both the phosphorylation and stabilization of transfected wt *MLK3* were blocked by CEP-1347 (Fig. 2A).

We extended our *MLK3* transfection experiments to d/n and wt *MLK1*, *MLK2*, and *DLK* and obtained similar results (Fig. 2B to D). In each case, the wt form showed an electrophoretic mobility shift and was expressed at substantially higher levels. Moreover, these effects were blocked by CEP-1347. Activation of transfected wt *MLKs* was confirmed by induced phosphorylation of endogenous JNKs, which in turn was inhibited by CEP-1347 (Fig. 2C and data not shown; see also Fig. S1 in the supplemental material). Taken together, these findings support a model in which *MLK* proteins are stabilized by apoptotic stimuli acting by a mechanism that requires *MLK* activation.

Next, we asked whether any one *MLK* family member would affect the expression of itself and that of other family members. Transfected EE-tagged *MLK3* had a detectable effect on the stability of Flag-*MLK3* (Fig. 3A) and on that of *MLK1* (Fig. 3B), *MLK2* (see Fig. 6A, below), and *DLK* (data not shown). Moreover, d/n *MLK3* blocked the stabilization not only of wt *MLK3* (Fig. 3A) but also that of wt *MLK1* (Fig. 3B) and wt *DLK* and wt *MLK2* (data not shown). Similar observations were made regarding the ability of additional wt and d/n *MLKs* to respectively stabilize or destabilize other family members (Fig. 3C and D and data not shown; see also Fig. S2 in the supplemental material). Such mutual effects on stability explain why overexpression of a single d/n *MLK* family member effectively blocks death evoked by other wt *MLKs* or by trophic factor deprivation (30).

Expression of d/n *MLKs* is elevated by coexpression of wt *MLKs*. Although our data indicate that *MLK* stabilization requires *MLK* activity, they do not establish whether individual *MLK* molecules must be active to be stabilized. To address this, we cotransfected tagged d/n *MLK2* with tagged wt *MLK1* or wt *MLK3* under conditions in which the levels of the d/n were not sufficient to block *MLK1* or *MLK3* activation. As shown in Fig. 3C, the wt *MLKs* greatly enhanced expression of d/n *MLK2*. The d/n protein also underwent a mobility shift similar to that observed for phosphorylated active *MLKs*. Similar results were achieved with other combinations of d/n and wt *MLK* forms (data not shown). Thus, an individual *MLK* molecule does not require kinase activity to be stabilized by the activation of other *MLKs*.

Activation of MKKs and JNKs is required for elevation of *MLK* proteins. MKK4 and MKK7 are major substrates for *MLKs* that, when phosphorylated and activated by *MLKs*, phosphorylate and activate JNKs to induce apoptotic death (4, 8, 20, 21, 25). To investigate whether *MLK* stabilization by apoptotic stimuli is direct or requires actions of their downstream targets, we cotransfected wt *MLKs* with d/n forms of *MKK4* or *MKK7* that block apoptosis evoked by *MLK* overexpression (30). We found that d/n *MKK7* reduced the expression and mobility shift/phosphorylation of each of the *MLKs*, as well as their ability to stimulate JNK phosphorylation (Fig. 3D and 4A and data not shown; see also Fig. S2 in the supplemental material). d/n *MKK4* promoted similar effects, though to a lesser degree (Fig. 4A and data not shown). These observations thus indicate that *MLK* stabilization requires MKK4/7 activity. Finally, also in agreement with our model of MKK activity-dependent *MLK* stabilization, cotransfected *MKK4* and *MKK7* stabilized d/n *MLK1* and d/n *MLK2* with

FIG. 3. MLKs regulate each other's stability. Different tagged wt or d/n *MLK* family members in the pCMS.EGFP vector were cotransfected into 293 cells with different tagged wt or d/n *MLK* family members or d/n *MKK7* in pCDNA3, or pCDNA alone as indicated. Cell lysates were analyzed by Western immunoblotting for levels of MLK3 with Flag antibody, MLK1 with His antibody, or d/nMLK2 with HA antibody as indicated. Where indicated, blots were reprobed with phospho-JNK (P-JNK) antibody to detect P-JNK, with GFP antibody as a control for transfection efficiency, and with JNK and/or ERK1 antibody as loading controls.

efficacies proportional to their abilities to induce JNK phosphorylation (Fig. 4B and C).

The involvement of MKKs in MLK stabilization led us to test whether the stabilization may also require JNK activity. To this end, we first used SP600125, a selective JNK inhibitor (2, 7). As shown in Fig. 1E, this drug effectively blocked the elevation of endogenous MLK3 induced by camptothecin. SP600125 also led to destabilization of overexpressed MLK1, MLK2, and MLK3 and caused these proteins to migrate with faster electrophoretic mobility (Fig. 5A). Finally, as an apparent consequence of its destabilizing effect on MLKs, SP600125 also suppressed the capacity of transfected MLKs to induce JNK phosphorylation (Fig. 5A and data not shown). In contrast, neither SB203580, a selective inhibitor of p38, nor U0126, a selective inhibitor of MEKs, fully blocked stabilization of transfected MLK1, -2, and -3 (Fig. 5A and data not shown). There was a partial effect of SB203580; this could be due to direct, nonspecific inhibition of JNK and/or to a role of p38 in this pathway via MLK-promoted activation of MKK3/6 (25). The various inhibitors (SP600125, SB203580, and U0126 as well as CEP-1347) had similar effects on induction of endogenous MLK3 camptothecin (Fig. 5A, lower right panel).

As an additional means to assess the role of JNKs in stabilization of MLKs, *MLK3* was cotransfected together with either the wild-type or a kinase-inactive d/n mutant of *JNK1*. As shown in Fig. 5B, cotransfection of wild-type *JNK1* with *MLK3*

somewhat enhanced the expression of MLK3 (Fig. 5B, upper panel) while, in contrast, the d/n kinase-dead form of *JNK1* significantly reduced its expression. Cotransfection with d/n *JNK1* also reduced expression of MLK1 (Fig. 5B, lower panel).

To further confirm the requirement for JNKs in stabilization of MLK3, we created two hairpin loop constructs that produce small interfering RNAs (siRNAs) targeted against both *JNK1* and *JNK2* transcripts. To assess the efficacy of the constructs, they were transfected into 293 cells, which were then cotransfected the next day with the siRNA constructs and *Flag-JNK1*. One day following the second transfection, the levels of endogenous *JNK1/2* and of Flag-tagged *JNK1* were determined. Both constructs down-regulated expression of endogenous and exogenous JNKs (Fig. 5C). The enhanced suppression of exogenous *JNK1* expression compared to that of endogenous JNKs (80% versus 50%) most likely reflects the likelihood that most of the cells transfected with *Flag-JNK1* are cotransfected with the siRNA constructs, whereas in the case of endogenous JNKs, only a portion of the cell population was transfected (approximately 85% in this experiment).

We next assessed the effect of suppressing JNK levels on MLK3 expression. As shown in Fig. 5C (right panels), cotransfection of *Flag-MLK3* with the two JNK siRNAs led to a significant fall of Flag-MLK3 expression compared to that in cells cotransfected with a control siRNA. To more fully evaluate the role of JNKs in stabilization of MLKs under apoptotic

FIG. 4. Involvement of MKK4/7 in the regulation of MLK protein phosphorylation and stability. **A.** Down-regulation of MLK2 phosphorylation and protein stability by d/n MKK4 and d/n MKK7. HA-tagged *MLK2* in the pCMS.EGFP vector was cotransfected into 293 cells with pCDNA3, d/n *MKK4*, or d/n *MKK7* in pCDNA3, as indicated. Cell lysates were prepared 24 h later and analyzed by Western blotting with HA antibody for the levels of MLK2 and for JNK and phospho-JNK as indicated in prior figures. Here and in subsequent panels, blots were probed with anti-GFP as a control for transfection efficiency. **B.** MKK4 and MKK7 induce the protein stability of d/n MLK1. His-tagged *MLK1* in the pCMS.EGFP vector was cotransfected into 293 cells with pCDNA3.Myc-MKK4 or Myc-MKK7 in pCDNA3, as indicated. Cell lysates were prepared 24 h later and analyzed by Western blotting with His antibody for the levels of MLK1 and Myc antibody for MKK4 or MKK7. **C.** Activation of multiple JNK pathway components elevates stability of d/n MLK2. HA-tagged kinase-dead d/n *MLK2* in the pCMS.EGFP vector was cotransfected into 293 cells with pCMS.EGFP, or separately with *MKK4*, *MKK7*, *POSH*, or *MLK3* in the same vector, as indicated. Cell lysates were prepared 24 h later and analyzed for the level of d/n MLK2 with HA antibody by Western blotting.

conditions, we transfected 293 cells with JNK siRNAs and 48 h later treated them with 10 μ M camptothecin for an additional 6 h, and then we used immunohistochemistry and Western blotting to evaluate levels of endogenous JNKs and MLK3 (Fig. 5D). As noted earlier, in response to camptothecin, there was a large increase in the proportion of cells in which MLK3 could be detected by immunostaining (Fig. 5D and Table 1). However, for camptothecin-treated cultures transfected with JNK siRNAs, most cells that lost detectable JNK expression (87% of cells) also lost detectable induction of MLK3 (Fig. 5D and Table 1). In contrast, almost all cells that retained JNK expression under such conditions (and were likely to be non-transfected) also showed induction of MLK3 (Table 1). These immunostaining results were complemented by Western blot analysis, which revealed that the siJNKs not only down-regulated total JNK levels in the cultures but also significantly diminished the elevation of endogenous MLK3 that occurred in response to camptothecin. Taken together, these findings indicate that JNKs play a required role in stabilization of MLK3 in response to apoptotic stimuli.

POSH stabilizes MLKs and is in turn stabilized by activation of MLKs and the JNK pathway. We previously reported that the multidomain protein POSH is a scaffold for MLKs and promotes their activation, thereby inducing JNK activation and apoptosis (29). If our models for POSH function and for stabilization of MLKs are correct, then POSH overexpression should stabilize MLKs by promoting their activation and that of JNKs. Accordingly, we found that expression levels of MLK2 (Fig. 6A) and MLK1 (Fig. 6B) as well as that of d/n forms of MLK2 (Fig. 4C) and MLK3 and DLK (data not shown) were significantly elevated when cotransfected with *POSH*.

We next assessed the possibility that endogenous POSH plays a required role in MLK stabilization. To test this hypothesis, we generated cell lines (293 siPOSH cells) in which levels of overexpressed POSH as well as elevated endogenous POSH expression induced by camptothecin were knocked down by constitutive expression of POSH siRNA (Fig. 6C, upper panels) (Z. Xu et al., submitted for publication). The 293 siPOSH and wt 293 cells were then transfected with *Flag-MLK3* in pCMS.EGFP. As shown in Fig. 6C (lower left panel), the expression of MLK3 was significantly lower in 293 siPOSH cells than in control 293 cells. Similar results were obtained with other MLK family members (MLK1 and MLK2) (data not shown). Furthermore, we found by immunohistochemistry and Western blotting that although expression and phosphorylation/activation of endogenous MLK3 were elevated in 293 cells treated with camptothecin, they were not detectably induced in 293 siPOSH cells (Fig. 6C, lower middle and right panels). These findings thus indicate that POSH plays an essential role in the stabilization of MLK3 (and apparently, other MLK family members) that occurs under apoptotic conditions.

Our previous observation that POSH is degraded by the proteasomal pathway (29) led us next to determine whether POSH levels are also regulated under proapoptotic conditions. As shown in Fig. 6D, expression of endogenous POSH is rapidly elevated in PC12 cells in response to camptothecin treatment and after trophic factor deprivation. Thus, POSH, along with MLKs, is elevated in response to apoptotic stimuli. To further distinguish whether the elevation of POSH was due to

FIG. 5. Involvement of JNKs in the regulation of MLK protein phosphorylation and stability. A. JNK inhibitor SP600125 down-regulates MLK phosphorylation, activity, and protein stability. Flag-MLK3, HA-MLK2 or His-MLK1 in the pCMS.EGFP vector was transfected into 293 cells. Four hours after transfection, cells were treated with CEP-1347, SP600125, SB203580, or U0126 as indicated. Cell lysates were prepared 24 h later and analyzed by Western blotting for levels of MLK1 with His antibody (upper left panel), MLK2 with HA antibody (upper right panel), and MLK3 with Flag antibody (lower left panel). Where indicated, here and in subsequent panels, blots were probed with phospho-JNK (P-JNK) antibody

TABLE 1. Downregulation of JNK levels by siRNA suppresses induction of MLK3 expression by camptothecin^a

JNK and MLK3 expression	% of cells with indicated response among:			
	si control	si control with camptothecin	siJNKs	siJNKs with camptothecin
JNK ⁺ /MLK3 ⁺	2	81	1	12
JNK ⁺ /MLK3 ⁻	97	14	12	1
JNK ⁻ /MLK3 ⁻	1	2	86	87
JNK ⁻ /MLK3 ⁺	0	3	1	0

^a Cells were transfected and treated as for Fig. 5D. Photos of several random fields were taken, and all cells were scored for simultaneous expression of JNKs and MLK3. Numbers are percentage of total cells scored (more than 200 cells were scored for each condition).

either transcriptional or posttranscriptional events, we co-treated PC12 cells with camptothecin and the transcriptional inhibitor actinomycin D. As shown in Fig. 6E, actinomycin D had a very limited effect on elevation of POSH protein, thus indicating that POSH levels, like those of MLKs, are largely regulated through a posttranscriptional mechanism.

We next examined whether the JNK pathway itself plays a role in stabilizing POSH, as in the case of MLKs. We first examined whether POSH overexpression (which is sufficient to activate the pathway) might regulate its own stability. Flag-tagged wt *POSH* was cotransfected with myc-tagged wt *POSH* or with a mutant *POSH* (Δ Ring *POSH*) that is defective in putative E3 ligase activity (and that is consequently more stable) but that is capable of promoting MLK and JNK activation as well as cell death (29). As shown in Fig. 7A, both wt and Δ Ring *POSH* enhance expression of coexpressed wt Flag-*POSH*. One potential mechanism for this self-stabilizing action is by activating MLKs and the JNK pathway. In support of this, the expression of transfected wt *POSH* was substantially reduced in the presence of CEP-1347 or SP600126 (Fig. 7B). Conversely, POSH expression was greatly elevated by coexpression with MLK family members (Fig. 7C and D). This stabilization was not mimicked by d/n MLKs and was significantly blocked by CEP-1347 and SP600125 (Fig. 7C and D) or after cotransfection of d/n MKK7 (Fig. 7E). Thus, POSH is stabilized by activation of the JNK pathway and is destabilized by inhibiting the pathway at multiple points. Moreover, the capacities of JNK pathway activators to elevate the levels of

cotransfected POSH driven by an exogenous promoter indicate that this effect occurs by a mechanism dependent on protein stabilization rather than on regulation of transcription. Finally, CEP-1347 (Fig. 7B) and SP600126 (data not shown) failed to diminish expression of transfected mutant Δ Ring *POSH*. This is consistent with the interpretation that turnover of wt POSH requires its own ring finger-dependent E3 ligase activity and that the ring finger mutant, because it does not turn over as readily under either viable or proapoptotic conditions, is therefore not subject to regulation by the JNK pathway.

Activation of the JNK pathway elevates expression of JIPs. JIP1 to -3 are an additional family of scaffold proteins that bind components of the JNK pathway and promote their activation (28, 32). We recently found that POSH directly associates with JIPs to form a proapoptotic multimolecular complex (PJAC, for *POSH*- and *JIP*-associated complex) that includes MLKs, MKK4/7, and JNKs (unpublished data). Past work indicated that UV exposure elevates endogenous JIP3 and decreases its electrophoretic mobility (9). These findings raised the possibility that expression of JIPs may also be subject to regulation by the JNK pathway. In support of this, cotransfection with *MLKs* significantly elevated the expression of all three JIP family members (Fig. 7E and F and data not shown). JIP stabilization was also suppressed by treatment with CEP-1347 and SP600125 and by cotransfection with d/n MKK7 (Fig. 7E and F and data not shown). Confirming these transfection studies, levels of endogenous JIP1 in neuronal PC12 cells increased after exposure to the apoptotic agent camptothecin (Fig. 7G).

Taken together, our data favor a model in which activation of the apoptotic JNK pathway promotes the stabilization of multiple pathway components, including MLKs, POSH, and JIPs.

DISCUSSION

Suppression and activation of the apoptotic JNK pathway: role of regulated protein expression. Cellular regulation of the death-promoting JNK pathway requires at least two levels of control: one is to render the constitutively expressed components of the pathway inactive when cell viability is desired.

to detect P-JNK, with GFP antibody as a control for transfection efficiency, and with ERK1 antibody as loading control. For the experiment shown in the lower right panel, PC12 cells were pretreated with CEP-1347, SP600125, SB203580, or U0126 as indicated for 2 h and then with 10 μ M camptothecin. Cell lysates were prepared 6 h later and analyzed by Western blotting for levels of MLK3 with MLK3 antibody and reprobed with ERK1 as loading control. B. d/n JNK1 interferes with MLK phosphorylation and protein stability. Flag-tagged *MLK3* (upper panel) or His-tagged *MLK1* (lower panel) in the pCMS.EGFP vector were cotransfected into 293 cells with pCDNA3 or kinase-inactive *JNK1* (d/n *JNK1*) in pCDNA3, as indicated. Cell lysates were prepared 24 h later and analyzed by Western blotting for levels of MLK3 and JNK1 with Flag antibody or MLK1 with His antibody. C. JNKs are required for protein stabilization of MLK3. siRNA against luciferase (siLuc) and two different siRNAs against both JNK1 and JNK2 (siJNK-A and siJNK-B) in the pSIREN-RetroQ-dsRed vector were separately transfected into 293 cells. One day later, the same cultures were cotransfected with the same siRNA constructs and either pCDNA.Flag-JNK1 and pCMS.EGFP (left panel) or pCMS.EGFP.Flag-MLK3 (right panel). One day later, cell lysates were analyzed by Western immunoblotting for levels of Flag-MLK3 and Flag-JNK1 with Flag antibody or for endogenous JNK1 and JNK2 with JNK antibody (right panel). Relative levels of signals were analyzed by scanning followed by quantification using the NIH software ImageJ. Flag-JNK1 levels were normalized for transfection efficiency with EGFP in the left panel, while JNK levels (right panel) were normalized with ERK1. Flag-MLK3 levels (right panel) were normalized for transfection efficiency with EGFP. D. JNKs are required for induction of endogenous MLK3 by DNA damage. siRNA against luciferase (siLuc) and siRNAs against both JNK1 and JNK2 in the pSilencer 2.1-U6 neo vector were transfected into 293 cells separately. Forty-eight hours later, cells were treated or mock treated with camptothecin for 6 h. Cells were then fixed and stained with antibodies against MLK3 (green) and JNK1/2 (red). Phase images are shown in the lower panel (left). A parallel experiment was performed by analyzing cell lysates by Western immunoblotting for levels of MLK3 with MLK3 antibody and JNKs with JNK antibody. The blot was reprobed with ERK1 as a loading control (right panel).

FIG. 6. POSH plays an essential role in stabilization of MLKs. A and B. POSH regulates the protein stability of MLK2 and MLK1. *HA-MLK2* (A) or *His-MLK1* (B) in the pCMS.EGFP vector was cotransfected into 293 cells with pCMS.EGFP, Flag-tagged *POSH*, or wt *MLK3* in the same vector as indicated. Cell lysates were prepared 24 h later and analyzed by Western immunoblotting for levels of MLK2 with HA antibody, MLK1 with His antibody, and phospho-JNK with P-JNK antibody. The blot was stripped and reprobed with Flag antibody for POSH and MLK3. Here and in subsequent panels, blots were probed with GFP antibody as a control for transfection efficiency and with ERK1 and/or JNK antibody as loading controls. C. POSH is essential for the stabilization of both exogenous and endogenous MLK3. Two independent clones of 293 cells were prepared (numbers 9 and 24) in which POSH expression is knocked down by constitutive expression of POSH siRNA (POSH⁻ cells). Upper left panel: comparison of expression of transfected Flag-POSH in wt and POSH⁻ cells. Upper right panel: comparison of expression of endogenous POSH in wt and POSH⁻ cells with or without exposure to camptothecin for 6 h. The experiment in the lower left panel shows that expression of transfected MLK3 and pMLK3 (24 h after transfection in pCMS.EGFP and probed with anti-P-MLK3 and anti-MLK3) is markedly lower in POSH⁻ cells compared with wt cells, as are levels of endogenous P-JNK. Lower middle panel: endogenous P-MLK3 and MLK3 levels in wt and POSH⁻ cells after 6 h with or without camptothecin exposure. Note that P-MLK3 or MLK3 is not elevated in response to camptothecin in the POSH⁻ cells. Where indicated, blots were reprobed with anti-JNK or anti-ERK1 as controls for loading and with anti-EGFP as a control for

Since overexpression or activation of any element of the pathway leads to JNK activation and cell death (29, 30), the maintenance of cellular viability is highly dependent on keeping the levels of pathway components low and their potentially lethal actions thus in check. A second level of control involves mechanisms that can be employed to rapidly activate the pathway in response to apoptotic stimuli in order to efficiently trigger death. The present findings clarify one aspect of these issues by showing that the stability of pathway components are subject to regulation with destabilization under conditions of viability and stabilization during apoptotic conditions. Moreover, we find that the system works as a feedback loop in that activation of the JNK pathway itself leads to stabilization of several of its key members.

Our study revealed that endogenous MLK3 levels substantially rise in response to various apoptotic stimuli and that this requires activation of the JNK pathway. Past work indicated an elevation of endogenous DLK triggered by DNA damage or growth factor deprivation (30). We further observed that apoptotic stimuli elevate endogenous levels of other JNK pathway members, including POSH and JIP1 proteins. Apoptotic stimulus-promoted elevation of endogenous MLK3 occurred in the presence of actinomycin, indicating a transcription-independent mechanism. This conclusion was further supported by studies using exogenous MLKs driven by a constitutive promoter in which we found that expression was elevated by apoptotic stimuli and JNK pathway activation. Similar experiments with transfected constructs further support protein stabilization mechanisms for the elevations of POSH and JIP proteins that occur in response to apoptotic stimuli and JNK pathway activation. In the case of endogenous POSH, this was further supported by an experiment carried out in the presence of actinomycin. Although we confirmed that Bim phosphorylation is induced by activation of the JNK pathway (11) through cotransfection of *Bim* in pCMS.EGFP with MLKs, we did not find that such conditions induced Bim stabilization (data not shown). These observations rule out the possibility that JNK pathway activation nonspecifically stimulates the cytomegalovirus promoter and support the selectivity of MLK, POSH, and JIP stabilization. Finally, our stabilization findings were made in a variety of cell types and apoptotic stimuli, thus indicating the generality of this mechanism.

Taken together, our observations thus provide a model in which viable cells are protected from the potentially lethal activities of JNK pathway proteins (especially POSH and MLKs) by promoting their destabilization to levels below that required to trigger death. In response to apoptotic stimuli, the pathway proteins are stabilized and rise to sufficiently high levels to activate the pathway and drive apoptosis.

A self-amplifying feed-forward loop model for activation of the JNK pathway. Examination of the conditions required for the stabilization of JNK pathway molecules revealed that blockade of any downstream element by pharmacological or genetic methods interfered with the stabilization of upstream proteins. In each experimental setting, stabilization of JNK pathway proteins ultimately required JNK activity and the participation of downstream molecules, including POSH, MLKs, and MKK4/7. These findings suggest a stabilization-based, self-amplifying feed-forward loop model to explain activation of the JNK pathway in an apoptotic environment (Fig. 8). In this model, JNK pathway proteins (especially the upstream elements POSH and MLKs) are unstable in viable cells as long as apoptotic JNK activity is low. In viable cells, the levels of key pathway proteins lie below the threshold required for induction of cell death. As a result, cell survival is maintained. Apoptotic stimuli, in contrast, ultimately lead to stabilization of pathway proteins, resulting in their accumulation to levels sufficient to activate the pathway and to trigger cell death. One could imagine that initial activation of any of the key pathway components will result in low-level JNK activation, which in turn would lead to increased stabilization of proximal molecules in the POSH-MLK-MKK4/7-JIP-JNK cascade. Starting from there, an amplification loop may be initiated that results in increased stabilization and activation until the threshold is ultimately reached to trigger induction of apoptosis.

Another aspect of JNK activation to be considered is that it does not always lead to death but may also serve other cellular functions (5). The feed-forward model may account for this dichotomy: POSH acts as a scaffold for MLKs, but it also interacts with MKK4/7 and JNKs (29), which in turn bind to JIP1. This raises the possibility that the feed-forward mechanism requires close proximity of its components within a single complex. If this were the case, then JNK activated outside of this complex might not effectively stabilize the pathway components.

One prediction of the self-amplifying model presented here is that inhibition of a single pathway component should prevent stabilization of additional pathway molecules. In consonance with this notion, inhibitors of MLK and JNK expression/activity and d/n forms of MLKs and MKK4/7 each prevent stabilization of multiple pathway components. Small-molecule inhibitors of the JNK pathway have been shown to block neuronal cell death in several different models (3) and have been proposed as potential therapeutic agents for a variety of maladies (3, 16). The capacity of single inhibitors to influence the entire pathway enhances their potential therapeutic effectiveness. Conversely, activation/stabilization of single proteins in the pathway leads to effective stabilization of other compo-

transfection efficiency. Right lower panel: parallel experiment in which camptothecin-treated wt and POSH⁻ cells were fixed and immunostained for endogenous MLK3 expression. No MLK3 expression was detected in the absence of camptothecin treatment (not shown). D. Endogenous POSH is elevated by camptothecin treatment and NGF deprivation. Neuronal PC12 cells were treated with 10 μ M camptothecin or deprived of NGF for the indicated times. Cell lysates were analyzed for POSH expression with POSH antiserum. A 0.5- μ g aliquot of total cell lysate from 293 cells transfected with pCMS.EGFP.Flag-POSH was loaded in the far right lane (5) as a positive control. The blot was stripped and reprobed with JNK antiserum as loading control. E. CEP-1347 and SP600125, but not actinomycin D, block camptothecin-elevated expression of endogenous POSH. PC12 cells were pretreated with CEP-1347 (200 nM), SP600125 (20 μ M), or 0.1 μ g/ml actinomycin D for 2 h before treatment with 5 μ M camptothecin for 6 h. Cell lysates were analyzed for POSH expression with POSH antiserum by Western immunoblotting. The blot was stripped and reprobed with ERK1 antiserum as a loading control.

FIG. 7. The MLK family regulates the protein stability of POSH and JIP1 via the JNK pathway. A and B. POSH regulates its own stability through the JNK pathway. (A) Flag-tagged wt *POSH* in the pCMS.EGFP vector was cotransfected into 293 cells with *pRK5* or separately with myc-tagged wild-type or the ring finger deletion form (Δ Ring) of *POSH* in *pRK5* as indicated. Cell lysates were prepared 24 h later and analyzed for the level of Flag-POSH with Flag antibody by Western immunoblotting. The blot was stripped and reprobed with Myc antibody to detect myc-POSH or myc- Δ Ring POSH. (B) Flag-tagged wt *POSH* or Δ Ring *POSH* in the pCMS.EGFP vector was cotransfected into 293 cells. At 4 h after transfection, cells were treated with CEP-1347 or SP600125 as indicated. Cell lysates were prepared 24 h later and analyzed for the level of Flag-POSH or Flag- Δ Ring POSH by Western immunoblotting with Flag antibody. C and D. The MLK family regulates the protein stability of POSH via the JNK pathway. Flag-tagged *POSH* in the pCMS.EGFP vector was cotransfected into 293 cells with pCDNA3 or separately with wt or d/n forms of *MLK1*, *MLK2*, or *DLK* in pCDNA3 (C) or with wt or d/n forms of *MLK3* in pCDNA3 (D), as indicated. CEP-1347 and the JNK inhibitor SP600125 were added to the medium 4 h after transfection, as indicated. Cell lysates were prepared 24 h later and analyzed for the levels of POSH with Flag antibody by Western blotting. Here and in subsequent panels, blots were probed with GFP antibody as a control for transfection efficiency. E. Stabilization of POSH and JIP1 induced by MLK3 can be suppressed by d/n MKK7. Flag-tagged *POSH* and *JIP1* were cotransfected into 293 cells with pCDNA3 or pCDNA3.MLK3, with or without pCDNA3.d/nMKK7, as indicated. Cell lysates were prepared 24 h later and analyzed by Western immunoblotting for expression of Flag-tagged proteins. F. Regulation of JIP1 stability by MLK and JNK activation. Flag-tagged *JIP1* in the pCDNA3 vector and 0.5 μ g of pCMS.EGFP were cotransfected into 293 cells with pCDNA3 or separately with wt or d/n forms of *MLK1*, *MLK2*, or *DLK* in pCDNA3, as indicated. CEP-1347 and SP600125 were applied as indicated. Cell lysates were prepared 24 h later and analyzed for the levels of JIP1 with Flag antibody by Western immunoblotting. G. Endogenous JIP1 is elevated by camptothecin treatment. Neuronal PC12 cells were treated with 10 μ M camptothecin for the indicated times. Cell lysates were analyzed for JIP1 expression with JIP1 antiserum. The blot was stripped and reprobed with lamin A antiserum as a loading control.

nents of the pathway and to death. This may represent a useful strategy for the design of effective proapoptotic drugs.

A major issue raised by our model is the means by which the destabilization of proteins in the JNK pathway is promoted in

viable cells. Previous findings support a role for AKT1 in this process. AKT1 has been shown to bind to JIP1 and to inhibit its interaction with JNK pathway kinases (10). This action does not require AKT activity and involves competition between

FIG. 8. Model for a feed-forward loop mechanism regulating activation of the JNK pathway by protein stabilization and destabilization.

JNKs and AKT for binding to JIP1 (10). AKT1 also interacts with and phosphorylates MLK3 and inhibits MLK3-mediated JNK activation (1). AKT2 has been found recently to bind POSH and may affect the interaction between MLK3 and POSH (5a). It is, therefore, intriguing to consider whether AKTs might be involved in blocking the feed-forward loop, leading to the destabilization of the JNK pathway components in viable cells. A related and presently unanswered issue is the means by which JNK activation feeds into the stabilization mechanism. It remains to be seen whether the stabilized proteins are themselves direct JNK targets or whether stabilization involves one or more intervening proteins that are regulated by JNK-dependent phosphorylation.

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