A sequential pathway (the JNK pathway) that includes activation of Rac1/Cdc42, mixed lineage kinases, MAP kinase kinases 4 and 7, and JNKs plays a required role in many paradigms of apoptotic cell death. However, the means by which this pathway is assembled and directed toward apoptotic death has been unclear. Here, we report that propagation of the apoptotic JNK pathway requires the cooperative interaction of two molecular scaffolds, POSH and JIPs. POSH (plenty of SH3s) is a multidomain GTP-Rac1-interacting protein that binds and promotes activation of mixed lineage kinases. JIPs are reported to bind MAP kinase kinases 4/7 and JNKs. We find that POSH and JIPs directly associate with one another to form a multiprotein complex, PJAC (POSH-JIP apoptotic complex), that includes all of the known kinase components of the pathway. Our observations indicate that this complex is required for JNK activation and cell death in response to apoptotic stimuli.

The c-Jun N-terminal/stress-activated kinases (JNKs) appear to play major roles in mediating apoptotic cell death of both neuronal and non-neuronal cells (1). A cascading pathway has been identified that leads to JNK activation in response to apoptotic stimuli. In this cascade, the GTP-bound form of Rac-1 and/or Cdc42 initiate a sequence of kinase phosphorylations/activations consisting of the mixed lineage kinases (MLKs), MAP kinase kinases (MKKs) 4 and 7 and JNKs (2–5). JNKs activated in this manner then promote death by phosphorylation/activation of the transcription factor c-Jun (1, 6) and/or the BH3-only domain protein BIM (7, 8).

POSH (plenty of SH3 domains) was identified as a binding partner of GTP-Rac1 (9), and its overexpression causes the death of both non-neuronal (9) and neuronal cells (10) by activating the JNK pathway. Consistent with a required role in apoptotic death paradigms, both antisense RNA and small interfering RNA directed against POSH transcripts protect neuronal PC12 cells and sympathetic neurons from death caused by NGF withdrawal (10). Moreover, antisense directed to POSH interferes with the elevation of intracellular phospho-Jun levels in NGF-deprived cells, thus implicating endogenous POSH in activation of JNKs and consequent c-Jun phosphorylation. The in vivo importance of POSH in cell death and in the apoptotic JNK pathway was recently demonstrated by a study in which knockdown of POSH by

infusion of antisense oligonucleotides lead to neuroprotection from cerebral ischemia as well as attenuated activation of the JNK signaling pathway (11).

Although many individual components of the apoptotic JNK pathway have been uncovered, questions remain about the molecular means by which they are brought into contact in cells and by which they are directed toward induction of death. Here, we show that the major components of the pathway are assembled in a multiprotein complex anchored by two interacting molecular scaffolds, POSH and JNK (JNK-interacting protein), and that formation of this complex is required for JNK activation and induction of apoptosis.

**EXPERIMENTAL PROCEDURES**

Plasmids—GST-JIP-1 was constructed by cloning the PCR product of mouse JIP-1 with primers 5′-gaattcgcctgcgattct-3′ and 5′-gatctcagtgctgttcctcct-3′ into the EcoRI and Sall sites of pGEX-KG. Full-length GST-POSH was constructed by cloning the PCR product of pPKS-MYC-POSH (10) with 5′-gaattcgcctggttgggctctg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′ into the BamHII and EcoRII sites of pGEX-KG. pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3 was constructed by PCR amplification of ΔRing-POSH, using primer pairs 5′-catatgcggtgaagtctgcgtgc-3′ and 5′-catatgcagctggcagcagctctc-3′, or 5′-catatgcagctggcaagcgagcgcg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′, cut with Ndel and Ndel/EcoRI, respectively, and cloned into the Ndel/EcoRI site of pCMS-EGF-FLAG-ΔRing-POSH. pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3 was constructed by PCR amplification of pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3, with primers 5′-catatgcagctggcaagcgagcgcg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′, cut with Ndel and Ndel/EcoRI, respectively, and cloned into the Ndel/EcoRI site of pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3, with primers 5′-catatgcagctggcaagcgagcgcg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′, cut with Ndel and Ndel/EcoRI, respectively, and cloned into the Ndel/EcoRI site of pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3, with primers 5′-catatgcagctggcaagcgagcgcg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′, cut with Ndel and Ndel/EcoRI, respectively, and cloned into the Ndel/EcoRI site of pCMS-EGF-FLAG-ΔRing-POSH ΔSH3-3, with primers 5′-catatgcagctggcaagcgagcgcg-3′ and 5′-gatctcagtgcgtcgggtcagccgcaccaccagcaccacactgta-3′, cut with Ndel and Ndel/EcoRI. 

**Protein Purifications, Immunoprecipitations, Pull-down Assays, Western Blotting, and Subcellular Fractionation**—GST-JIP-1 and GST-POSH were purified as described (10). JIP-1 protein was obtained by 3 h of thrombin cleavage of GST-JIP-1 bound to beads, followed by centrifugation and repeated dialysis against phosphate-buffered saline. Protein labeling, pull-down assays, immunoprecipitations, and Western blotting were performed as described previously (10). For subcellular fractionation, cells were incubated on ice for 5 min in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Heps, pH 7.5) and spun at low speed (2000 × g) to separate fractions.

**Cell Culture and Transfections**—Culture and transfections of PC12 cells and 293 cells were as described previously (10).

**Immunoreagents**—POSH antiserum was raised in rabbits using mouse POSH peptide AA253-363 as the immunizing antigen (Covance, Cambridge, MA)
Inc.). JIP-1 antibodies were from Santa Cruz (sc-15353) and BD Biosciences (611891). Anti-phospho-JNK (9252), anti-lamin A (2032), and anti-phospho-MLK-3 (2811) were from Cell Signaling. Rac1 was from BD Biosciences (R56220), ERK and c-MYC were from Santa Cruz (sc-93 and sc-789-G, respectively), anti-FLAG (A2220) and anti-MYC (A7470) beads were from Sigma, as was antibody for FLAG (F7425). The secondary antibody was AlexaFluor 568 from Molecular Probes.

Immunostaining—Cells were grown on a glass coverslips and fixed with 4% paraformaldehyde at 4 °C for 10 min followed by treatment with 0.5% Triton X-100 in phosphate-buffered saline for 10 min. For staining, antibodies were used as indicated in figure legends and text. Slides were visualized with a Nikon Eclipse E800 microscope.

RESULTS

Studies designed to define the role of POSH in JNK signaling indicated that POSH directly interacts with MLKs and that it serves as a scaffold for MLK activation (10). We also noted in reciprocal immunoprecipitation experiments that POSH interacts with additional downstream members of the JNK pathway including MKK-4, MKK-7, and JNKS (10). This raised the possibility that either POSH alone may serve as a scaffold for the entire JNK activation cascade or that it couples to downstream components via one or more intermediates. To distinguish between these possibilities, we carried out in vitro pull-down assays with purified full-length GST-POSH and in vitro translated 35S-labeled JIP-1, MKK-4, MKK-7, and MLK-3 (a positive control). Although the predicted binding to MLK-3 was observed, there was no detectable binding to any of the other proteins (Fig. 1A). These observations thus suggested the existence of at least one intermediate component(s) between POSH and downstream pathway kinases.

Members of the JIP (JNK-interacting protein) family have been identified as additional prominent participants in the JNK pathway that interact with MLKs, MKK-4, and JNKS (12–14). Like POSH, JIPs appear to play a key role in JNK-mediated death. For instance, neurons isolated from JIP-1 null mice do not show JNK activation or apoptotic death in response to stimuli such as excitotoxic and anoxic stresses (15). It was thus logical to hypothesize that JIPs may mediate the interaction of
POSHP with MKKs and JNKs. To address this, we performed a series of co-immunoprecipitations by transfecting 293 cells with Myc epitope-tagged POSH and FLAG-tagged JIP-1, JIP-2, or JIP-3. Cell lysates were subjected to immunoprecipitation with anti-Myc, and the immunocomplexes were probed for FLAG-tagged JIPs. All three JIPs were detected in the immunocomplexes of co-transfected cells and were absent from immunoprecipitates of cells transfected with JIPs in the absence of Myc-POSH (Fig. 1B, upper panel). Reciprocal co-immunoprecipitations revealed that Myc-POSH was present in complexes with all three FLAG-JIPs, but was not immunoprecipitated in the absence of co-transfected JIPs (Fig. 1B, lower panel).

To further characterize the Poshi-JIP interaction, we tested the ability of the GST-POSH fusion protein to pull down in vitro translated JIPs. All three JIPs (as well as the positive control MLK-3) bound to GST-POSH, but not to GST (Fig. 1C). Thus, the interaction between Poshi and JIPs appears to be direct.

We next assessed the hypothesis that JIPs mediate the indirect association of MKK-4/7 and JNKs with Poshi. GST-POSH was immobilized on glutathione-coated beads and preincubated with or without purified JIP-1 protein. Unbound protein was removed by washing, and the beads were incubated with in vitro translated [35S]methionine-labeled MKK-4, MKK-7, or JNK-1. After further extensive washing, the bead-associated proteins were analyzed by SDS-PAGE and fluorography. MKK-4, MKK-7, and JNK-1 were recovered from the beads only when they were preincubated with JIP-1 (Fig. 1D). No labeled protein was recovered from similarly treated beads without attached GST-POSH (data not shown). These findings strongly support the model that JIP-1 associates with Poshi and bridges the interaction of Poshi with MKK-4/7 and JNK-1.

Finally, to determine the stoichiometry of Poshi/JIP-1 interaction, 293 cells were co-transfected with a small amount of Flag-JIP-1 plasmid and an excess of Flag-POSH plasmid. Lysates were immunoprecipitated with anti-JIP-1 antibody, resolved by Western blotting, and probed for both tagged proteins with anti-FLAG (Fig. 2A). This revealed a ratio of ~1:1. Similar results were achieved when the plasmid concentrations were reversed and lysates were immunoprecipitated with anti-POSH antibody (data not shown).

The association of Poshi with JIPs in vitro and under conditions of overexpression raised the questions of whether the endogenous proteins also interact in cells and, in particular, whether they do so in the presence of apoptotic stimuli. To address this, neuronal (NGF-treated) PC12 cells were exposed to the DNA-damaging agent camptothecin for various times, and immunoprecipitates of cell lysates obtained with anti-JIP-1 were analyzed for the presence of Poshi. Previous studies have established that camptothecin evokes apoptotic death of neuronal PC12 cells and neurons by ~16–24 h of treatment (16–19). As described elsewhere (20), the endogenous levels of Poshi, MLK-3, and JIP-1 increase under apoptotic conditions. Significantly, endogenous Poshi and MLK-3 co-immunoprecipitated with endogenous JIP-1. Such association was present both before and after camptothecin treatment with increasing levels of each component in the complex in approximate proportion to their increased total cellular levels of expression (Fig. 2B). There was no signal in any case when anti-JIP-1 was omitted from the immunoprecipitations (data not shown). We also detected increased levels of MKK-7 and JNK in immunocomplexes with JIP-1 after camptothecin exposure, although the total cellular levels of these kinases were unchanged under these conditions. This most likely reflects the enhanced levels of JIP-1 in camptothecin-treated cells, although we cannot rule out a change in binding constants between MKK-7 and JNKs and JIP-1.

We also assessed the fractions of total cellular endogenous Poshi, MLK-3, MKK-7, and JNK that associated with JIP-1 in immunoprecipi-
Mutant forms of POSH deficient in JIP-1 binding fail to induce apoptosis or to promote phosphorylation of JNK but retain the capacity to promote phosphorylation of MLK-3. A, mutational analysis of POSH domains involved in association with JIP-1. Full-length immobilized GST-JIP-1 was incubated with indicated 35S-labeled in vitro translated deletion mutant forms of POSH. After extensive washing, bound radioactive proteins were resolved and visualized by SDS-PAGE followed by fluorography. Domains of wild type (WT)-POSH are depicted beneath the fluorograph. Zn, zinc Ring finger domain; box, rac1 binding domain. B, FLAG-tagged ΔRing-Posh and the ΔRing-ΔSH3/4-Posh were cotransfected with MYC-tagged MKK-7 or JNK-1 as indicated. Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG, and the Western immunoblots were probed with anti-MYC. C, POSH mutants defective in interaction with JIP-1 fail to induce apoptosis. ΔRing-POSH, ΔRing-ΔSH3/4-POSH, ΔRing-ΔSH3/4-POSH, and ΔRing-Δ61-136AAΔSH3/4-POSH cloned into pCMS-EGFP were transfected into neuronally differentiated PC12 cells in the presence or absence of 200 nM CEP-1347, a selective JNK pathway inhibitor (3). Total numbers of GFP-positive cells in each culture (in 24-well dishes) were counted 24 h later. To normalize for potentially variable transfection efficiencies of each construct, in each case the
tates after camptothecin exposure by comparing scanned Western blot signals from the immunoprecipitates and immunoprecipitate supernatant fractions (taking total volumes and gel loading into account). This indicated that essentially all cellular POSH associated with JIP-1. Moreover, the fractions of total cellular MLK-3, MKK-7, and JNK present in the immunocomplexes were ~28, <10, and <12%, respectively. These finding thus indicate that considerable fractions of cellular POSH and MLK-3 are associated with JIP-1 in apoptotically stimulated neuronal PC12 cells and that, in contrast, only a subtraction of total cellular proportions of MKK-7 and JNKs associate with this complex. The relatively low total cellular proportions of MKK-7 and JNKs recovered in co-immunoprecipitates with JIP-1 could reflect either that these molecules are in molars excess to JIP-1 or that their binding constants favor dissociation under the conditions of our experiments.

To determine whether endogenous POSH and JIP-1 interact in an alternative paradigm of cell death, neurally differentiated PC12 cells were subjected to withdrawal of NGF, a condition that results in apoptosis within 18–24 h. In this case too, POSH co-precipitated with JIP-1, and there was an increase in levels of the complex by 4–6 h of NGF withdrawal that was in rough proportion to the increases in cellular levels of both proteins (Fig. 2C). Thus, it appears that POSH and JIP-1 interact both before and after apoptotic stimuli and that apoptotic stimuli increase the cellular levels of the complex by enhancing the cellular levels of both of its principal components.

The association of POSH with JIPs coupled with the interactions of POSH with MLKs and of JIPs with MKK-4/7 and JNKs suggests the formation of a functional complex containing all the known kinase components of the JNK pathway. To evaluate this model, we investigated the domains of POSH that are responsible for its interaction with JIP-1. To do so, we employed a direct GST pull-down assay in which GST-JIP-1 was used as a bait to detect interaction with various POSH deletion mutants. Mutants with deletions of SH3 domains 1 and/or 2, the rac1 binding domain or the zinc Ring finger did not show impaired interaction with GST-JIP-1 (data not shown). In contrast, as shown in Fig. 3A, sequential deletion of the highly homologous C-terminal SH3 domains number 3 and number 4 of POSH greatly reduced interaction with GST-JIP-1. We observed in past that SH3 domains 3 and 4 are not required for interaction of POSH with MLKs nor for MLK phosphorylation and activation (10). Deletion of a small region of POSH between the zinc finger domain and first SH3 domain (corresponding to amino acids 61–136) also led to a significant decrease in association of POSH with GST-JIP-1 (Fig. 3A). Finally, deletion of all three regions of POSH (i.e. SH3 domains three, four, and amino acids 61–136) led to a substantial loss of the interaction with GST-JIP-1 (Fig. 3A). As shown below, despite its impaired interaction with JIP-1, this POSH mutant retains its capacity to activate MLK-3, thus indicating that the mutant protein has not undergone a wholesale change in conformation.

If, as our data indicate, JNKs and MKKs interact with POSH via their association with JIP-1, then POSH mutants with impaired JIP-1 binding properties would display a weaker interaction with MKks and JNKs in vivo. Because POSH is a zinc Ring finger containing putative E3 ligase and is rapidly degraded in cells via the proteasomal pathway (10), we carried out our studies with POSH constructs (∆Ring-POSH) in which the zinc Ring finger was deleted. This assured that the various constructs were adequately and equally expressed. Fig. 3E provides an example of the approximately equal expression of such constructs after transfection into 293 cells. Near equal expression was also confirmed in neuronal PC12 cells (data not shown). The ∆Ring-POSH retains its capacity to bind MLKs, activate the JNK pathway, and to promote cell death (10). FLAG-tagged ∆Ring-POSH and the ∆Ring-∆SH#3/4/POSH were co-transfected with MYC-tagged MKK-4/7 or JNK-1/2, and cell lysates were subjected to immunoprecipitation with anti-FLAG, and the resulting Western immunoblots were probed with anti-MYC. Fig. 3B shows that ∆Ring-∆SH#3/4/POSH has a greatly reduced association with MKK-7 and JNK-1 when compared with its wild type counterpart. Comparable results were achieved with MKK-4 and JNK-2 (data not shown). The residual association that was detected most likely reflects the low degree of association that occurs between ∆SH#3/4-POSH and JIP-1 (Fig. 3A).

Next, we assessed the functional consequences of interfering with the association of POSH with JIP-1. Past work has established that overexpression of full-length POSH or ∆Ring-POSH is sufficient to promote cellular apoptosis (9, 10) and that POSH knockdown is protective from death (10). ∆Ring-POSH and various ∆Ring-POSH deletion mutants impaired for interaction with JIP-1 were transfected into neuronal PC12 cells, which were subsequently scored for survival. In contrast with full-length ∆Ring-POSH, the mutants with highly impaired JIP-1 interaction (∆Ring-∆SH#3/4 and ∆Ring-∆AA61-136/∆SH#3/4) showed little if any significant killing, whereas the ∆Ring-∆SH#3 mutant, which is somewhat more effective in binding JIP-1, evoked an intermediate level of death (Fig. 3C). We observed in past that there is a substantial synergism between MLKs and POSH in the induction of cell death, presumably because of the capacity of POSH to promote MLK activation (10). As shown in Fig. 3D, MLK-2 was substantially less effective in promoting death of neuronal PC12 cells when co-transfected with ∆Ring-POSH ∆AA61-136/∆SH#3/4 than when co-transfected with the full-length ∆Ring construct. Similarly, MLK-2 failed to synergize with ∆Ring-POSH ∆AA61-136/∆SH#3/4 in generation of phospho-JNK (Fig. 3D).

Our findings suggest a model in which POSH and JIP complement one another in an apoptotic complex. In this model, POSH binds and promotes activation of MLKs, which then induce sequential phosphorylation/activation of MKK-4/7 and JNKs, which are brought to the complex via interaction with JIPs. If this is correct, then POSH forms that are defective for JIP interaction should be able to evoke MLK phosphorylation/activation, but should be impaired with respect to phosphorylation/activation of JNKs. To evaluate this, we transfected 293 cells with the above POSH constructs and 24 h later assessed the levels
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of phospho-MLK-3 and phospho-JNK (Fig. 3E). In consonance with our model, although each of the constructs induced similar levels of MLK-3 phosphorylation, ΔRing-POSH ΔSH3#3/4 and ΔRing-POSH ΔAA61-136/ΔSH3#3/4 showed greatly reduced capacity to induce phospho-JNK. As in the above JIP-1 binding and apoptotic assays, ΔRing-POSH ΔSH3#3 induced an intermediate level of JNK phosphorylation. Thus, POSH mutants that are defective for interaction with JIP-1 but that retain the capacity to bind and activate MLKs are greatly impaired in inducing cell death and JNK phosphorylation, both alone and in synergism with an MLK.

An additional prediction of our model is that POSH mutants with impaired capacity to interact with JIPs should act as partial antagonists to endogenous POSH and should provide protection form apoptotic stimuli. To test this, neuronal PC12 cells were transfected with empty vector, wild type POSH, or POSH ΔSH3#3/4 and 18 h later were subjected to NGF withdrawal. As shown in Fig. 3F, POSH ΔSH3#3/4 provided a significant protection from death evoked by NGF deprivation. This contrasted with wild type POSH, which did not show protection and which elevated death in the first day after NGF withdrawal.

It has been shown that JIP-1 localizes in the neurites and cytoplasm of cultured hippocampal neurons and translocates to the perinuclear compartment of such cells in response to oxygen-glucose deprivation (15). Given the interaction of POSH and JIP-1, we therefore wished to determine the localization of POSH in cells affected by apoptotic stimuli. We first used immunostaining to localize endogenous POSH and JIP-1 in neuronal PC12 cells before and after exposure to apoptotic stimuli. Because the only suitable antisera available to us were prepared in rabbit, each antigen was separately localized. Consistent with the study cited above, JIP-1 immunostaining was at highest levels at punctate locations in neurites of control cells with a lower level of diffuse cytoplasmic expression (Fig. 4, i and iii); in response to camptothecin treatment, intense JIP-1 staining was lost from neurites, and the protein showed a more intense, largely perinuclear distribution (Fig. 4, ii and iv). In control cells, POSH exhibited a widespread punctate pattern in neurites and somatic cytoplasm (Fig. 4v). After camptothecin treatment, as in the case of JIP-1, the largest increase in immunostaining was observed in the perinuclear region (Fig. 4vii). A similar change in staining pattern was observed after NGF deprivation (data not shown). Thus, in response to apoptotic stimuli, both JIP-1 and POSH accumulate in the perinuclear region.

To further examine whether its interaction with JIP plays a role in the redistribution of POSH that occurs in response to apoptotic stimuli, we turned to biochemical fractionation. Lysates of neuronal PC12 cells (treated with or without camptothecin for various times) were prepared with Triton X-100 and subsequently separated into “cytoplasmic” (Rac1-containing) and crude “nuclear-associated” (Lamin A-containing) fractions (see “Experimental Procedures”). An analysis of endogenous POSH revealed that it was present in both fractions in viable cells and that the largest increase occurred in the crude nuclear fraction after camptothecin exposure, particularly after 4 h (Fig. 5A). This suggests that the POSH seen in the perinuclear region by immunostaining largely associates with the crude nuclear fraction. We also determined the relative levels of JIP-1 in our fractions. As in the case of POSH, there was a large increase in JIP-1 within the crude nuclear fraction by 4–6 h of camptothecin treatment (Fig. 5A). Thus, based on both immunostaining and cell fractionation, both POSH and JIP-1 accumulate in the perinuclear area in response to an apoptotic stimulus.

Next, we assessed the subcellular distribution of our various ΔRing POSH constructs after expression in 293 cells (Fig. 5B). Transfected ΔRing-POSH, which, like camptothecin, is an apoptotic stimulus, was mainly present in the nuclear-associated fraction. In contrast, ΔRing-POSH ΔSH3#3/4 and ΔRing-POSH ΔAA61-136/ΔSH3#3/4 were mainly expressed in the cytoplasmic fraction. ΔRing-POSH ΔSH3#3 exhibited an intermediate level of distribution between the two fractions. Thus, perinuclear translocation of POSH appears to require the capacity for association with JIP-1. In support of this, similar findings were obtained when POSH constructs were co-expressed with JIP-1. That is, under such circumstances, the majority of ΔRing-POSH was recovered from the nuclear fraction, whereas most of ΔRing-POSH ΔSH3#3/4 was present in the cytoplasmic fraction (Fig. 5B).

Finally, we determined whether JIP-1 translocation is dependent on POSH and/or POSH interaction. In the absence of co-expressed POSH, transfected JIP-1 was observed entirely in the cytoplasmic fraction (Fig. 5C). However, in the presence of either ΔRing-POSH or ΔRing-POSH ΔSH3#3/4 a substantial level of JIP-1 was recovered from the nuclear fraction (Fig. 5C). Thus, ΔRing-POSH ΔSH3#3/4, which neither localizes to the nuclear fraction nor binds well to JIP-1, nevertheless promotes JIP-1 translocation. This suggests that JIP-1 translocation does not require direct interaction with POSH but rather that it may be dependent on a POSH-mediated action.

FIGURE 4. Camptothecin promotes the relocalization and perinuclear accumulation of JIP-1 and POSH. Neuronal PC12 cells treated without or with 20 μM camptothecin for 6 h were immunostained with anti-JIP-1 (i and ii, red; iii–iv, black and white) or anti-POSH (v and vi, red), and nuclei were stained with Hoescht 33258 (blue). Panels i and ii show immunostaining and nuclei in color. Arrows in i show the accumulation of JIP-1 protein in neuronal processes prior to camptothecin treatment. Panels iii and iv show the same images in black and white and with the contrast and brightness of the entire images adjusted to reveal the somatic and neuritic margins. Arrows in iv show the outline of neurites.
DISCUSSION

Although many individual components of pathways that lead to activation of JNKs have been described, questions remain about how these are brought together and about how they are directed toward various JNK actions such as the promotion of apoptosis. In the present work, we have focused on one well described apoptotic pathway in which sequential activation of the kinases MLK and MKK-4/7 lead to activation of JNKs and consequent cell death. Collectively, our findings support a model (depicted in Fig. 6) in which propagation of this pathway depends on the cooperative interaction of two scaffold proteins, POSH and JIP. Within this multiprotein complex, for which we propose the name PJAC, POSH binds and activates MLKs, which leads in turn to sequential activation of MKK-4/7 and JNKs that are recruited to the complex by JIPs. Our data further indicate that the ratio of POSH to JIP in such complexes is 1:1.

A number of observations support this model. We found that POSH and JIP complex with one another in vitro and that both the exogenously expressed and endogenous proteins interact with one another in living cells. Although POSH has been found to directly bind MLKs and to interact with MKK-4/7 and JNKs, our data reveal that the association with MKK-4/7 and JNK is indirect and requires the presence of JIPs. Moreover, mutant forms of POSH that retain the capacity to bind and activate MLKs, but that bind only poorly to JIPs, show a greatly reduced interaction with MKK-4/7 and JNK-1 and are deficient in promoting JNK phosphorylation/activation. Such findings support the model that POSH and JIP play complementary roles in PJAC with the former recruiting and activating MLKs and the latter delivering MKK-4/7 to the complex as a substrate for MLKs.

Although our findings support a role for PJAC in cell death, they do not indicate that the complex is specifically formed in response to apoptotic stimuli. The complex was detectable in healthy cells but at relatively low levels. Our present and past data show that apoptotic stimuli lead to a considerable elevation of cellular levels of POSH, MLKs, and JIP-1 via protein stabilization (20) and that this in turn is reflected by a large increase in cellular PJAC levels. Thus, it appears that cell death is driven by the increase in PJAC levels because of elevation of its components rather than to any major effect of apoptotic stimuli on formation of the complex per se.

JIPs have been reported to directly bind MLK family members (12).
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However, our past (10, 20), and present findings indicate that the presence of POSH and its interaction with JIPs are required for efficient JNK activation. For example, overexpression of JIP alone does not drive JNK activation and only does so when co-expressed with the MLKs (5). Of further relevance, we noted a large reduction in MLK-induced JNK phosphorylation in cells with knocked down POSH expression (20), seen in Fig. 6C. Finally, it appears that at least in the case of the MLK family member DLK, only the inactive form of the enzyme directly binds JIPs (21).

Past and present work also supports a role for PJAC in cell death as well as in JNK activation. Forced expression of POSH induces cell death via activation of MLKs, MKK-4/7, and JNKs, whereas knockdown of JIP to form the PJAC complex is ineffective in promoting death on its own or in synergizing the proapoptotic activity of MLK-2. Several studies have also underscored the role of JIP-1 in cell death. Whitmarsh et al. (15) reported that neurons (both in vivo and in vitro) from animals null for JIP-1 expression showed marked reduction in JNK activation and death in response to excitotoxic stress and oxygen-glucose deprivation. In another study, the brains of JIP-1 null animals, in comparison with wild type controls, did not show sustained JNK activation and had much smaller infarct volumes in response to a transient focal ischemic insult.

Our findings indicate that apoptotic stimuli promote several changes in the PJAC complex. For one, as discussed above, the total level of cellular PJAC increases because of stabilization and elevated expression of several of its components. Presumably, this change plays a role in promotion of JNK activation and of cell death. In addition, our observations indicate that both JIP-1 and POSH accumulate in the perinuclear region. Such a translocation has been previously described for JIP-1 (14). The mechanism by which this occurs is unclear, but past work indicates that JIPs bind to microtubule-associated motor proteins and that this regulates their intracellular distribution (22). The increase in total cellular PJAC levels may also contribute to the enhanced perinuclear accumulation of the complex. Our data indicate that perinuclear accumulation of POSH requires its association with JIPs, but not vice versa. Thus, consistent with its known association with motor proteins, the JIP component of the complex appears to be responsible for the change in subcellular distribution. The functional role of this redistribution is presently unknown. One possibility is that the presence of JIPs and POSH, and presumably of PJAC, in the perinuclear region may facilitate the proapoptotic phosphorylation of c-Jun. Activation of MLKs and JNKs has been associated with a variety of cellular functions in addition to induction of death (1). Our findings raise the possibility that scaffolds such as POSH and JIPs may modulate the functional activities of these enzymes by preferentially directing them to specific cellular targets or compartments. In case of PJAC, these activities appear to be associated with induction of cell death.

Acknowledgments—We thank Dr. R. Davis (UMASS) for providing JIP constructs, R. Townley (Columbia University) for his help with generation of POSH peptide for production of antisera, and Drs. Carol M. Troy and Dmitry Goryunov (Columbia University) for their assistance with microscopy.

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