Proapoptotic Nix Activates the JNK Pathway by Interacting with POSH and Mediates Death in a Parkinson Disease Model

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Nix, a pro-apoptotic BH3-only protein, promotes apoptosis of non-neuronal cells, although the mechanisms involved remain incompletely understood. Using a yeast two-hybrid screen with POSH (plenty of SH3 domains, a scaffold involved in activation of the apoptotic JNK/c-Jun pathway) as the bait, we identified an interaction between POSH and Nix. Co-immunoprecipitation and in vitro binding studies confirmed a direct interaction between POSH and Nix in mammalian cells. When overexpressed in HEK293 cells, Nix promotes apoptosis along with enhanced phosphorylation/activation of JNKs and their target c-Jun. These effects appear to be dependent on POSH because Nix does not promote either JNK/c-Jun phosphorylation or apoptosis of 293 cells that do not express POSH. Nix and POSH appear to mutually stabilize one another and this effect could contribute to their promotion of death. Past work showed induction of Nix transcripts in a cellular model of Parkinson disease based on neuronal PC12 cells exposed to 6-hydroxydopamine. Here, we confirm elevation of Nix protein in this model and that Nix overexpression causes apoptotic death of PC12 cells by a mechanism dependent on c-Jun activation. Expression of s-Nix, a dominant-negative form of Nix, protects neuronal PC12 cells from 6-hydroxydopamine but not from nerve growth factor deprivation. These results indicate that Nix promotes cell death via interaction with POSH and activation of the JNK/c-Jun pathway and that Nix protein is induced and contributes to cell death in a cellular model of Parkinson disease.

The Bcl-2 superfamily of proteins is subdivided into three, distinct, functional groups (1). The anti-apoptotic family, which includes Bcl-2 and Bcl-XL, contain Bcl-2 homology (BH)3 BH1, BH2, BH3, and BH4 domains. The pro-apoptotic BH3-containing proteins consist of multidomain proteins such as Bad and Bax, which contain BH1–3 domains, and BH3-only proteins such as Bim and Bid. The 19 kDa-interacting protein BNip3 and its homologue Nix (BNip3L/BNip3L/B5) form a subfamily of proapoptotic BH3-only proteins (2). These proteins, initially identified by yeast two-hybrid screening for cellular proteins that bind the anti-apoptotic adenoviral E1B 19-kDa protein (3), promote apoptosis in a number of non-neuronal cell types (2, 4–6). However, the mechanisms by which these proteins promote apoptosis remain unclear and are likely distinct from those of other BH3-only proteins.

As with several other BH3-only proteins, Nix (2) and BNip3 (7) localize to mitochondria through hydrophobic, putative membrane-spanning domains at their COOH termini. This region also permits them to form stable homo- and heterodimers with other Bcl-2 family members (8). Interestingly, although the BH3 domain in Nix and BNip3 is functionally equivalent to the BH3 domain in Bax (7), deletion of this domain only partially protects cells from Nix- or BNip3-induced apoptosis (7, 8). However, BNip3 mutants lacking the transmembrane domain do not localize to the mitochondria and do not promote apoptosis (9). Furthermore, a naturally occurring, short form of Nix, sNix, which lacks the transmembrane domain acts as a dominant-negative and protects cells from apoptosis induced by Nix (5). Thus, the transmembrane domain appears to be critical for the proapoptotic function of Nix and BNip3, while the BH3 domain does not.

Previous work has suggested that Nix and BNip3 promote apoptosis by different mechanisms. Although recombinant Nix directly promotes cytochrome c release from isolated mitochondria (6), BNip3 causes early plasma membrane permeabil- ity and late changes in mitochondria (10). Thus, Nix and BNip3 seem to promote death through both necrotic and apoptotic mechanisms. The Caenorhabditis elegans orthologue of BNip3 promotes activation of the caspase orthologue CED-3 (11). Similar to the case for mammalian BNip3 and Nix, promotion of death by ceBNip3 again depends on the transmembrane domain and occurs independently of the BH3 domain.

The abbreviations used are: BH, Bcl-2 homology; 6-OHDA, 6-hydroxydopamine; siRNA, small interfering RNA; NGF, nerve growth factor; JNK, c-Jun NH2-terminal kinase; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; WT, wild type; IP, immunoprecipitation.
The mitogen-activated protein kinases enable cells to respond to numerous extracellular signals with a variety of responses. Previous work has identified an important role for the c-Jun NH₂-terminal kinase (JNK)/c-Jun pathway in apoptosis following various stimuli including hypoxia-ischemia (12, 13), 6-hydroxydopamine (6-OHDA) treatment (14), and withdrawal of trophic support (15). Recent work has uncovered a key role for the scaffold protein POSH (plenty of SH3 domains) in regulating the JNK/c-Jun pathway (16). POSH binds to the mixed lineage kinases and promotes their activation (16). This in turn, via interaction of POSH with the scaffold JNK-interacting protein (17), leads to serial activation of MKK4/7 and the JNKs and ultimately to phosphorylation/activation of c-Jun and apoptotic death (16). Overexpression of POSH in multiple cell types promotes activation of the JNK/c-Jun pathway and apoptosis (16–18). Furthermore, knockdown of POSH using siRNA protects primed PC12 cells from nerve growth factor (NGF) deprivation (16) and antisense oligonucleotides against POSH inhibit JNK activation and apoptosis following cerebral ischemia (19). Additional studies indicate that POSH levels are very low in healthy cells and that in response to apoptotic stimuli, POSH protein is stabilized leading to accumulation of sufficient POSH levels to activate the JNK/c-Jun pathway and promote death (17).

Previous work has identified several ways in which JNK activation may regulate the synthesis and activities of various Bcl-2 family members (20–22). However, we are unaware of any reports of BH3-only proteins activating JNK to promote apoptosis. We report here that Nix binds to and stabilizes POSH and activates the JNK/c-Jun pathway. Moreover, death induced by Nix requires JNK/c-Jun activation and the presence of POSH. We also find that Nix plays a required role in a death paradigm (exposure of neuronal PC12 cells to the Parkinson disease mimetic 6-OHDA) previously shown to require activation of the JNK pathway (14). We show that 6-OHDA induces Nix protein in this experimental system and that inhibition of Nix by the naturally occurring dominant-negative, sNix protects against apoptotic death. This protection is specific to this death paradigm as sNix does not protect PC12 cells following NGF deprivation, a paradigm that we show does not induce Nix.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media RPMI 1640 and Dulbecco’s modified Eagle’s medium were obtained from Mediatech (Herndon, VA). Lipofectamine2000 was obtained from Invitrogen. Recombinant human NGF (hrNGF) was generously supplied by Genentech (South San Francisco, CA). Hoechst dye 33342 and anti-hrNGF antiserum were obtained from Sigma. Anti-JNK/phospho-JNK were from New England Biolabs (Beverly, MA). Anti-Nix was from ProSci (Poway, CA) and anti-FLAG and anti-Myc from Santa Cruz Biotechnology (Paso Robles, CA). Anti-FLAG beads and anti-Myc beads were from Sigma. SP600125 was purchased from Calbiochem. 6-Hydroxydopamine was from Sigma and was prepared as described previously (23). The cell-permeable, specific JNK inhibitor, DJNKI-1, was obtained from Alexis Biochemicals (San Diego, CA). Anti-POSH antibody (mouse polyclonal) was obtained from Abnova (Taipei City, Taiwan).

**Generation of Plasmids**—POSH constructs and dominant-negative cJun have been described previously (16). All other constructs were confirmed by direct sequencing at the Columbia Health Sciences DNA Facility using the appropriate primers. FLAG-tagged mouse Nix in the pcDNA3.1 vector was graciously provided by Dr. G. W. Dorn, University of Cincinnati. The cDNA insert was subcloned into PET21 at the Xbal and Hind3 sites. The insert was then digested using Xbal and XhoI and cloned into the multiple cloning site of pCMS-EGFP using Xbal and Sall (pCMS-EGFP.Nix). The FLAG-tagged dominant-negative sNix (pCMS-EGFP.sNix) was cloned using pCMS-EGFP.Nix as a template using the following primers: 5’-ACC ATG GGG GAC TAC AAG GAC GAC GAT GAC AAG-3’. DJNKI-1 was obtained from Alexis Biochemicals (San Diego, CA). Anti-POSH antibody (mouse polyclonal) was obtained from Abnova (Taipei City, Taiwan).

**Yeast Two-hybrid Screen, Immunoprecipitation, Western Immunoblotting, and in vitro Binding Assays**—The two-hybrid screen was performed with the ProQuest™ two-hybrid system, Invitrogen as described previously (18). Clones were selected for strong interactions based on screening for expression of reporter genes His, LacZ, and URA3. Co-immunoprecipitation and Western immunoblotting and in vitro binding assays were performed as described previously (16). All membranes were stained with Ponceau S prior to immunoblot to determine equivalent loading. Chemiluminescent signals were digitally acquired using a Kodak Image Station 4000MM and Kodak Molecular Imaging Software version 4.0.0. Bands were identified visually, fit to a Gaussian function, and relative intensity determined and reported where visual determination is not obvious. FLAG-Nix, FLAG-NixΔBH3, and FLAG-sNix were used for in vitro transcription and translation using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). GST-POSH was produced as described previously (16).
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**Determination of Cell Death**—Strip counting to determine cell survival was performed as described previously (16). Nuclear morphology was examined using Hoechst 33342 staining (15). The individual counting was blinded to the experimental groups in all cases. All cell counting was performed in triplicate and repeated a minimum of three times. Apoptotic ratios were performed on three separate experiments and the mean and standard differentiation determined.

**Statistics**—All pairwise comparisons were performed with unpaired t test with significance set at the p < 0.05 level. Unless specified, tests were two-sided. The Graph Pad In Stat program was used for these calculations. Comparisons of normalized, relative intensity were preformed using the single sample t test.

**RESULTS**

**Nix Binds the JNK Scaffold POSH**—The scaffold protein POSH promotes pro-apoptotic signaling via the JNK cascade by binding to several members of the pathway including mixed lineage kinases and JNK-interacting proteins (16). To identify additional POSH-binding partners, we performed a yeast two-hybrid analysis using POSH cloned into a bait vector as previously described (18). One clone corresponded to Nix, a pro-apoptotic BH3-only protein (2) that promotes cell death through mechanisms that appear distinct from other BH3-family members. Given the ability of POSH to promote apoptosis through JNK activation, we chose to further evaluate this interaction and its effects on JNK signaling.

We performed co-immunoprecipitation experiments to confirm interaction of Nix and POSH in mammalian cells. Full-length FLAG-tagged Nix in the pCMS-EGFP vector was co-expressed with Myc-tagged POSH lacking the RING domain (ΔZnPOSH) or with vector expressing only EGFP. This form of POSH was used because it is much more stable than the WT molecule but retains its capacity to activate the JNK pathway (16). Cell lysates were immunoprecipitated with anti-Myc, and the immunocomplexes were probed for tagged Nix. As shown in Fig. 1A, top, FLAG-Nix was detected only in immunoprecipitates from cells expressing Myc-ΔZnPOSH. The increased levels of Nix in lysates co-expressing ΔZnPOSH are due to stabilization of Nix by POSH (see Fig. 5, D and E). To rule out the possibility that the observed FLAG-Nix signal in the IP was due to elevated Nix expression in presence of ΔZnPOSH, the blots were overexposed. At more than 6-fold longer exposure (Fig. 1A, middle) Nix levels in the control IP are barely detectable. To further confirm a specific interaction, we also performed reciprocal immunoprecipitations using anti-FLAG. Fig. 1B shows that Nix and ΔZnPOSH again specifically co-immunoprecipitated under these conditions.

To confirm a direct interaction between POSH and Nix, we performed in vitro binding assays. Because previous work showed that Nix does not fully require its BH3 domain to promote cell death (7), we postulated that in addition to Nix, NixΔBH3 would interact directly with POSH. We also evaluated a naturally occurring dominant-negative isoform of Nix, sNix, which lacks only the terminal 10 amino acids of the protein and that, unlike Nix, is not targeted to mitochondrial membranes (5). Nix, NixΔBH3, and sNix were transcribed and translated as radiolabeled products in vitro and incubated with a GST-POSH fusion protein or empty GST beads as described under “Experimental Procedures.” As shown in Fig. 1C, POSH binds directly to all three of these proteins. Thus, Nix binds directly to POSH, and this binding is not dependent on either the BH3 domain (NixΔBH3) or on the COOH-terminal 10 amino acids of the protein (sNix).

**Nix Promotes Death and Activates the JNK Pathway**—Past studies indicate that Nix is induced in response to apoptotic stimuli such as hypoxia (28, 29) and, in cardiomyocytes, pressure overload hypertrophy (5) and that its elevated expression promotes apoptotic death. However, the mechanism by which Nix promotes apoptotic death has not been explored. Because Nix interacts with POSH, we postulated that it might promote JNK phosphorylation/activation and consequent apoptosis. We first examined the effect of Nix overexpression on the nuclear morphology of HEK293 cells transfected with pCMS-EGFP, Nix or pCMS-EGFP alone using the nuclear dye Hoechst 33342. Nix induced significant, apoptotic death of WT293 cells by 48 h post-transfection. Approximately 30% of Nix-transfected cells exhibited condensed, apoptotic nuclei compared with ~5% of cells expressing EGFP alone (Figs. 2A and 3). Next, we expressed Nix in 293 cells to determine whether JNK is activated. Immunoblot of cell lysates revealed a significant increase in levels of phospho-JNK in cultures transfected (efficiency ~80%) with Nix or NixΔBH3 (but not in cultures transfected with sNix (Fig. 2B). This level of total cellular JNK activation (1.5–2-fold) is similar to that which has been associated with death in other apoptotic paradigms (24). Furthermore, as shown in Fig. 2C, Nix and NixΔBH3 (but not sNix) promote...
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FIGURE 2. Nix promotes apoptosis and JNK activation in 293 cells. A, WT or siPOSH cells were transfected with the indicated constructs in the pCMS-EGFP vector. 48 h after transfection cells were stained with Hoechst 33342. The percentage of GFP+ cells with apoptotic nuclei was determined. Results are the mean ± S.D. for three separate experiments. Neuronally differentiated (primed). B, pCMS-EGFP, pCMS-EGFP.sNix, pCMS-EGFP.Nix, and pCMS-EGFP.NixΔBH3 were overexpressed in HEK293 cells. Lysates were collected 20 h later and immunoblot performed for p-JNK. The membrane was re-probed to detect total JNK levels. The intensity of the p-JNK bands relative to c-Jun in succession. C, lysates prepared as described for B were immunoblot for p-cJun and total cJun in succession.

FIGURE 3. Nix induces apoptosis in WT293 cells but not in cells lacking POSH. WT HEK293 cells or HEK293 cells constitutively expressing an siRNA-targeting POSH (siPOSH cells) were transfected with the indicated constructs. Nuclei were stained with Hoechst 33342 and photomicrographs taken 48 h after transfection. Images were taken at random locations without floating cells in the specific field. pCMS-EGFP.Nix increased the number of apoptotic WT293 cells versus pCMS-EGFP alone, but this was not the case in siPOSH cells (also see Fig. 2A). Red arrows indicate some of the apoptotic appearing cells with loss of polygonal morphology and condensed nuclei.

POSH Is Required for JNK Activation and Apoptosis Promoted by Nix—As our data revealed that Nix interacts with POSH and can promote JNK activation, we hypothesized that POSH might be required for Nix-induced JNK activation and apoptosis. To test this hypothesis we used a HEK293 cell line that permanently expresses a POSH siRNA (siPOSH cells) and that, as previously established (25), expresses reduced POSH protein (supplemental Fig. 2), even in response to stress stimuli.

Unlike WT293 cells (Fig. 2, B and C), siPOSH cells do not show elevated phosphorylation of JNK or c-Jun in response to Nix overexpression (Fig. 4, A and B). As shown below, overexpression of POSH stabilizes Nix. To confirm that Nix is appropriately expressed in cells lacking POSH, we examined expression of tagged pCMS-EGFP.Nix in WT and siPOSH cells as shown in Fig. 4C. When normalized to GFP, expression of Nix was only slightly reduced in siPOSH cells compared with WT cells. Thus, the differential effects of Nix in WT and siPOSH cells appear to be due to the absence of POSH from the latter and not to a deficiency of Nix expression.

To evaluate whether POSH is required for apoptosis induced by Nix, we compared the effect of Nix overexpression on apoptosis of WT and siPOSH cells. In contrast to the induction of apoptosis in WT cells, in siPOSH cells there was no significant difference between siPOSH cells transfected with either pCMS-EGFP.Nix or pCMS-EGFP (Fig. 2A). Representative images are shown in Fig. 3. Although the transfection efficiency of siPOSH cells is lower than WT293 cells (~70% versus 85%), only GFP+ cells were counted in these experiments. Similar results were obtained with NixΔBH3 (see supplemental Fig. 1), and as expected, sNix did not induce apoptosis in either WT or siPOSH cells (data not shown). Taken together, these data indicate that POSH is required for JNK activation and apoptosis induced by Nix.

Nix and POSH Reciprocally Stabilize One Another—POSH contains a RING domain that possesses E3 ligase activity (16). This domain regulates the turnover of POSH and other proteins through the ubiquitin-proteasome pathway and results in very low levels of POSH expression in healthy cells (16, 18). We have recently established that POSH is stabilized in response to apoptotic stimuli and that the consequent elevation of cellular POSH levels promotes JNK activation and cell death (17). We therefore questioned whether Nix may contribute to promoting the stabilization of POSH.

To test this, we co-expressed FLAG-tagged POSH in pCMS-EGFP with Nix, NixΔBH3, or sNix in the pcDNA vector or
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A.

![POSH & GFP](image)

B.

![SP600125](image)

C.

![Relative Density](image)

D.

![Flag-Nix, Flag-ΔBH3, Nix, POSH, α-Flag, α-GFP](image)

E.

![Flag-Nix, Flag-ΔBH3, POSH, α-Flag, α-GFP](image)

FIGURE 5. Nix and POSH stabilize each other. A, FLAG-tagged POSH in the pCMS-EGFP vector was expressed with the indicated cDNAs in the pcDNA3.1 vector. Lysates were collected 20 h later and subjected to immunoblot as indicated. Membranes were reprobed with GFP to determine equal transfection of POSH in each sample. B, parallel transfections to A were performed in the presence of the JNK inhibitor SP600125 (20 μM). C, relative intensity of POSH bands, normalized to GFP for immunoblots performed in A and B. Shown are the mean and S.D. for two consecutive experiments. Note that, as explained in the text, the effects of Nix on POSH are more dramatic in the presence of the JNK inhibitor. D, pCMS-EGFP.Nix or pCMS-EGFP.NixΔBH3 ("Flag-ΔBH3") were co-transfected with POSH in the pcDNA3.1 vector or empty vector as control. 20 h after transfection cells were collected and subjected to immunoblot as indicated. Shown are representative results of four experiments. Note that NixΔBH3 actually runs at a higher apparent molecular weight than Nix itself. E, parallel experiments to those in C were performed in the presence of SP600125 (20 μM).

empty vector as control. In the presence of Nix, POSH was apparently stabilized as shown in Fig. 5A. This experiment was repeated twice and the relative density of POSH to EGFP in each lane of the Western blot determined. Nix increased POSH levels by 2.2 ± 0.3-fold. sNix had little if any evident effect on POSH stabilization (1.3 ± 0.7-fold) (Fig. 4B). Our past findings indicated that POSH stabilization in response to several apoptotic stimuli is dependent on JNK activation (21). To determine whether this is so in the case of Nix, we repeated the experiment in the presence of the JNK inhibitor, SP600025. Fig. 5, B and C, shows that stabilization of POSH by Nix persists in the presence of JNK inhibition (2.07 ± 0.11-fold). We confirmed that the JNK inhibitor prevents c-Jun activation (phosphorylation) in these experiments by Western immunoblot of treated and untreated cells (data not shown).

Nix contains PEST sequences that may control its ubiquitin-independent processing by the proteasome (2), and Chen et al. (2) confirmed that lactacystin enhances the stability of Nix. To determine whether Nix expression is subject to regulation via its interaction with POSH, we co-expressed Nix and NixΔBH3 with Myc-POSH or empty vector in HEK293 cells. As shown in Fig. 5D, POSH enhanced the expression of co-expressed Nix and NixΔBH3 more than 3-fold. We next asked whether this stabilization depends on JNK activation as with other constituents of the POSH-JNK signaling pathway (17). When the same experiment was repeated in the presence of the JNK inhibitor SP600125, POSH-induced stabilization of Nix and NixΔBH3 persisted (Fig. 5E). Thus, the mechanism by which POSH stabilizes Nix appears to be distinct from that by which other constituents of the pathway are stabilized.

6-Hydroxydopamine Induces Apoptosis of Neuronal PC12 Cells Dependent on Nix—Our data suggest that elevation of Nix promotes JNK activation and apoptosis through an interaction with POSH and that this interaction leads to stabilization of Nix. Previous work has shown that 6-OH dopamine (6-OHDA) induces apoptosis of neurally differentiated (NGF-treated) PC12 cells and that this requires JNK activation (14). Furthermore, we previously reported that 6-OHDA treatment of such cells, which is a cellular model for Parkinson disease, results in a 12-fold induction of Nix mRNA levels as determined by serial analysis of gene expression (26). This model thus provides a potential system in which to test the role of Nix induction and of Nix in apoptotic death of neurons and to assess whether in this case also Nix acts via the JNK pathway.

We first determined whether Nix protein also increases in this model by subjecting neuronal PC12 cells to treatment with 6-OHDA followed by immunoblot with a commercially available Nix antibody. As shown in Fig. 6A, Nix levels began to increase by 4 h following 6-OHDA exposure and continued to increase up to 10 h, a time before overt signs of death in this model (26).

This finding raised the possibility that Nix may play a role in 6-OHDA-induced death. To test this, we next determined whether elevated Nix expression is sufficient to kill neuronal PC12 cells. PC12 cells were transfected with pCMS-EGFP, Nix or with pCMS-EGFP alone, and the transfected cells were assessed for survival. As shown in Fig. 6B, cells transfected with Nix exhibited reduced survival compared with cells expressing EGFP alone. To test whether JNK activation and its subsequent activation of c-Jun is required for Nix-induced apoptosis of neuronal PC12 cells, we co-expressed pCMS-EGFP. Nix or pCMS-EGFP alone with dominant-negative c-Jun and monitored the transfected cells for survival. Dominant-negative c-Jun restored survival of Nix-transfected cells to that of cells expressing only EGFP (Fig. 6C).

Because 6-OHDA elevates Nix levels and because Nix overexpression causes death of neuronal cells, we next queried whether Nix plays a required role in 6-OHDA-induced apoptosis. To do so, we transfected either pCMS-EGFP.sNix, a dominant-negative Nix isoform, or pCMS-EGFP alone, in neuronal PC12 cells and subjected them to 6-OHDA treatment 72 h after transfection. The transfected cells were then examined 24 h later for relative survival and apoptotic death. 6-OHDA induced a highly variable degree of cell death in individual experiments. Therefore, two representative survival experiments are shown in Fig. 6D. This study was repeated five times, and, for any given degree of death induced by 6-OHDA, cells expressing sNix always exhibited improved survival relative to control cells. A separate experiment confirmed that transfection with sNix did not alter cell survival under basal conditions without 6-OHDA (Fig. 6E), in keeping with previous data (5).

To confirm that sNix prevents apoptosis induced by 6-OHDA, we also evaluated nuclear morphology. Overexpres-
Elevated Nix levels play role in apoptosis induced by 6-hydroxydopamine. A, primed PC12 cells were maintained in NGF containing medium for a minimum of 5 days. They were then treated with 6-OHDA (50 μM) and collected at the indicated time points. Lysates were then immunoblotted for Nix; overexpressed, FLAG-tagged Nix was run in parallel as a positive control (data not shown). Membranes were stripped and reprobed for neurofilament light (NFL) to determine equal loading. This experiment was repeated three times with similar results. *, p < 0.02. B, 48 h after exposure to NGF, primed PC12 cells were transfected with pCMS-EGFP.Nix or empty pCMS-EGFP. The GFP + cells were counted daily, starting 24 h after transfection. Data were normalized to 100% survival in pCMS-EGFP-expressing cells. Cells overexpressing Nix exhibited reduced survival compared with control cells. Shown is one experiment, performed in triplicate. C, 48 h after priming, PC12 cells were co-transfected with the indicated plasmids. To ensure co-expression in EGFP + cells, empty CMV or dominant-negative (d/n) c-Jun was used at 4-fold excess relative to the EGFP-containing vector. Cells were counted as described for B, and survival was normalized to 100% for EGFP/dominant-negative c-Jun-expressing cells. D, 48 h after priming, PC12 cells were transfected with pCMS-EGFP.sNix or pCMS-EGFP. 72 h after transfection, cells were treated with 6-OHDA (50 μM). Cells were counted 1 h later and again the following day. Each experiment was performed in triplicate and the mean ± S.D. is shown. Two separate experiments are shown to indicate the variable levels of death in control cells. Despite this variability, sNix provided protection in all cases (five total experiments). One-tailed t tests were used to determine whether protection by sNix was statistically significant. E, we confirmed that sNix does not alter survival of primed PC12 cells not subjected to a death stimulus. 48 h after priming, PC12 cells were transfected with pCMS-EGFP.sNix or empty pCMS-EGFP vector. Cells were counted 24 and 48 h later as shown in the graph (mean ± S.D.). F, cells treated as described for D were stained with Hoechst 33342 and the percentage of GFP + cells with apoptotic nuclei determined 24 h after 6-OHDA treatment.

**DISCUSSION**

Since the identification of Bcl-2 as an anti-apoptotic protein, a great deal of work has elucidated the mechanisms by which BH3-family proteins regulate cell survival (1). Generally, the anti-apoptotic family members, which contain all four BH domains, localize to the mitochondria. There, through their interactions with pro-apoptotic BH3-proteins, they inhibit the loss of mitochondrial contents into the cytoplasm. Several of the pro-apoptotic family members have been shown to bind to and inhibit the anti-apoptotic BH3 proteins and thereby induce apoptosis. Other BH3 proteins promote apoptosis through direct effects on the mitochondrial membrane.

Previous work on Nix and BNip3 suggests that they promote apoptosis through different mechanisms than other BH3-only proteins. Nix and BNip3 both contain a BH3 domain that is functionally equivalent to that in Bax (7). Although this domain is required for heterodimerization with other BH3 family proteins, it is only partially required for induction of apoptosis by Nix and BNip3 (7, 8). Therefore, heterodimerization with anti-apoptotic BH3 family members cannot be the sole mechanism by which Nix/BNip3 promote death. Our work confirms that Nix lacking the BH3 domain causes apoptosis when overexpressed. The present findings indicate an alternative mechanism by which Nix can promote apoptotic death, namely by activation of the JNK/c-Jun pathway through its interaction with POSH. Overexpression of Nix increased the phosphorylation of JNKs and c-Jun and inhibition of the effects of JNK
using a dominant-negative c-Jun showed that JNK is required for apoptosis induced by Nix. Furthermore, in the absence of POSH, Nix was unable to activate JNK or to promote apoptosis. In our systems, the capacities of Nix to bind POSH and to phosphorylate/activate JNKs and c-Jun as well as to promote death were not dependent on the presence of its BH3 domain.

Nix appears to induce JNK activation at least in part through stabilization of POSH. Although the proteasome regulates levels of Nix (2), previous work has not identified an ability of Nix to regulate levels of other proteins. The mechanism by which this occurs is not entirely clear, but our data indicate that it does not depend on JNK activation in contrast to other stimuli that stabilize POSH (17). One possibility is that Nix binds to POSH, masking the RING domain and thereby makes POSH inaccessible to the proteasome. Alternatively, Nix binding might alter the subcellular localization of POSH making it inaccessible to the proteasome. Nix and NixΔBH3 exhibit mitochondrial localization (7, 8) and thus may also serve to recruit POSH and the activated JNK complex to mitochondria. Increasing evidence suggests that localization of the activated JNK cascade to the mitochondria is important to promote apoptosis (14, 22). Further work will be required to determine whether Nix can indeed recruit POSH and other JNK cascade proteins to mitochondria.

Although a significant amount of work has investigated the HIF-dependent regulation of Nix transcription (28, 29), little is known about the post-transcriptional regulation of Nix. Nix contains PEST sequences, which are known to play a role in promoting protein degradation, and so it may be rather unstable under basal conditions. Our findings indicate that overexpressed POSH enhances the stability of Nix and NixΔBH3. This suggests that the increase in Nix levels observed in response to certain apoptotic stimuli may result from both enhanced transcription and stabilization of the protein. One might imagine a situation in which an apoptotic stimulus induces Nix transcription and that the reciprocal stabilization of POSH and Nix activates a feed forward loop that elevates both proteins to levels sufficient to drive apoptotic death. Previous work from our laboratory has shown that POSH stabilizes constituents of the JNK pathway including mixed lineage kinase family members, MKK4/7 and JNK-interacting proteins and that this stabilization is dependent upon JNK activity (17). However, treatment of cells with the JNK inhibitor SP60025 had minimal effects on POSH-dependent stabilization of Nix or NixΔBH3, thus indicating a different mechanism of action.

Our work establishes a causative role for the Nix-JNK/c-Jun pathway in an in vitro model of Parkinson disease. Recent work has identified a role for the JNK pathway in 6-OHDA-induced death (14) as well as in other models of Parkinson disease (30), but the mechanism by which this is initiated has been unknown. Previous studies from our laboratory revealed that Nix mRNA levels increase following 6-OHDA treatment of neuronal PC12 cells (26). Here we show that Nix protein levels also increase following 6-OHDA treatment. Prior work has shown that Nix levels are up-regulated under hypoxic conditions (29), and the oxidative stress induced by 6-OHDA may be responsible for Nix induction under these conditions. However, Nix levels may be regulated by different mechanisms in different cell types or following different stimuli (31). Our data do not indicate whether up-regulation of Nix at the level of mRNA or by protein stabilization is more important in this model. In fact, both may be required to amplify the signal to sufficient levels to promote apoptosis. Additional studies will be required to establish the mechanism responsible for Nix protein stabilization and its relative importance in mediating death evoked by 6-OHDA and other apoptotic stimuli.

To test whether Nix plays a role in our cellular model of Parkinson disease, we used sNix and found that it protected primed PC12 cells from 6-OHDA. On the other hand, sNix provided little, if any, protection following NGF deprivation and no protection against death induced by the DNA damaging agent camptothecin. Interestingly, the POSH-dependent JNK pathway is known to play a required role in death induction in both of these models (16, 17). In contrast to 6-OHDA treatment, NGF deprivation does not induce an increase of Nix protein, which may explain its differential role in these paradigms. This suggests that, depending on the nature of the apoptotic stimulus, there are alternative mechanisms by which the POSH-dependent JNK/c-Jun pathway can be activated.

Collectively the present work identifies a novel mechanism by which Nix induces JNK activation and apoptosis through its interaction with POSH. Importantly this pathway plays a requisite role in 6-OHDA toxicity as an in vitro model of Parkinson disease. This pathway may provide an important target for therapeutic intervention in Parkinson disease and other diseases involving oxidative stress or mitochondrial dysfunction.

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