Inhibition of JNK by Overexpression of the JNK Binding Domain of JIP-1 Prevents Apoptosis in Sympathetic Neurons*

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Studies in non-neuronal cells show that c-Jun N-terminal kinases (JNK) play a key role in apoptotic cell death. In some neurons JNK is also thought to initiate cell death by the activation of c-Jun. JNK inhibition has been achieved pharmacologically by inhibiting upstream kinases, but there has been no direct demonstration that inhibition of JNK can prevent neuronal death. We have therefore examined whether the JNK binding domain (JBD) of JNK-interacting protein-1 (JIP-1, a scaffold protein and specific inhibitor of JNK) can inhibit c-Jun phosphorylation and support the survival of sympathetic neurons deprived of NGF. We show that expression of the JBD in >80% of neurons was sufficient to prevent the phosphorylation of c-Jun and its nuclear accumulation as well as abrogate neuronal cell death induced by NGF deprivation. JBD expression also preserved the capacity of mitochondria to reduce MTT. Interestingly, although the PTB domain of JIP was reported to interact with rhoGEF, expression of the JBD domain was sufficient to localize the protein to the membrane cortex and growth cones. Hence, JNK activation is a key event in apoptotic death induced by NGF withdrawal, where its point of action lies upstream of mitochondrial dysfunction.

The c-Jun N-terminal kinases (JNKs)1 belong to the mitogen-activated protein kinase (MAPK) family, sharing significant sequence homology with other members of the group that include MAPK and p38 kinase. JNK is activated by upstream kinases with phosphorylation in response to environmental stress, cytokines, apoptosis-inducing agents, or trophic factor deprivation and in turn phosphorylates the transcription factor c-Jun (1–3). In addition to c-Jun, JNK has several known substrates within the cell including activating transcription factor-2 (ATF-2), Elk-1 (4), bcl-2 (5), p53 (6), and DENN/MADD (7).

A number of studies have suggested that the activation of the JNK cascade and the subsequent phosphorylation of c-Jun may be a fundamental step in the induction of neuronal apoptosis (8–13). In vivo, the combined knockout of junk-1 and -2 genes show impaired developmental cell death in the CNS (14), whereas the hippocampus of junk-3 knockout mice show a reduced sensitivity to excitotoxic death coupled with a decrease in the level of stress-induced c-Jun phosphorylation (15). In culture, functional blockade of c-Jun by microinjection of antibodies or dominant negative c-Jun protects neonatal sympathetic neurons from NGF withdrawal-mediated neuronal cell death (10, 11). Moreover, when the c-Jun gene was replaced with a mutant c-Jun allele (serine residues at positions 63 and 73 (phosphorylation sites of JNK) mutated to alanine residues), the mutant mice, similar to the junk-3 knockout, were resistant to epileptic seizures, and hippocampal neuronal apoptosis induced by the excitatory amino acid kainate was inhibited (16). The induction of c-Jun expression is also observed following ischemia and in chronic neurodegenerative disease prior to apoptotic death (17). These data suggest that JNK is an important therapeutic target for prevention of apoptosis in the nervous system but until now, it has not been possible to inhibit JNK directly (25). Delineating the neuronal effects of JNK is important, because JNK has also been suggested to be required for axon regeneration (17).

Recently two cytoplasmic proteins that bind selectively to JNK but not to other related MAP kinases including p38 were identified as JNK-interacting proteins 1 and 2 (JIP-1 and JIP-2, Refs. 18 and 19). JIP-1/2 are scaffold proteins that bind to several components of the JNK signaling cascade including the JNK group of MAPKs, the MAPKK isoform MKK7, and members of the mixed-lineage kinase group of MAPKKKs (20). Overexpression of JIP-1 causes the cytoplasmic retention of JNK and thereby inhibits gene expression mediated by the JNK signaling pathway (18). However, interpretation of these results is complicated by the large number of proteins with which JIP-1 interacts. JIP-1 contains two well described domains; the C-terminal PTB domain of JIP-1 interacts with RhoGEF and may be involved in growth cone remodeling (21), consistent with the location of JIP-1 in nascent growth cones, whereas the JNK binding domain (JBD) of JIP-1 is located at the N terminus (18). We reasoned that expression of JBD should be an effective inhibitor of JNK and thus can be used to

JNK-interacting proteins; JBD, JNK binding domain of JIP-1; NGF, nerve growth factor; SCG, superior cervical ganglion; MAPK, mitogen-activated protein kinase; Ad, adenovirus; pfu, plaque-forming units; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; HRP, horseradish peroxidase; BAF, Boc.aspartyl(O-methyl)CH2F; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PTB, phosphotyrosine binding.

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§ The abbreviations used are: JNK, c-Jun N-terminal kinase; JIP,
Examine whether JNK inhibition is sufficient to prevent neuronal death. In this study, an adenoviral (Ad) vector expressing the JBD of JIP-1 has been used to investigate the importance of the activation of JNK in mediating cell death signals in primary sympathetic neurons. We report that JBD expression inhibited the phosphorylation of c-Jun and prevented apoptosis from occurring in superior cervical ganglion (SCG) cultures following the removal of NGF.

**EXPERIMENTAL PROCEDURES**

Recombinant Adenovirus Vectors—Recombinant E1-deleted Ad constructs were produced according to standard techniques (22). The FLAG-tagged JNK binding domain (JBD) of JIP-1 (residues 127–281) was cloned into the Ad transfer vector pXCCMV (A. Byrnes, Oxford University) under the control of the human cytomegalovirus major (CMV) IE promoter enhancer fragment (663 base pairs of pCMV 1; Invitrogen) followed by a bovine growth hormone poly(A) tail. Recombinant virus (Ad-Flag-JBD) was generated by homologous recombination with pM17 in 293 cells (ATCC, Manassas, VA), grown to high titer, and purified by CsCl density-gradient ultracentrifugation. The Ad-EGFP vector was constructed as described previously (23).

Sympathetic Neuronal Culture and Adenoviral Transfection Analysis—Sympathetic neurons from the SCG of neonatal rats were cultured either on coverslips or in 12-well plates precoated with polylysine/laminin in L15-CO2 medium supplemented with 3% rat serum, 20 μM uridine/5-fluorodeoxyuridine, and 20 ng/ml NGF. Neurons were infected after 4–6 h of plating with the respective viruses as previously described (23) to obtain an infection rate of 80–90% (about 100 pfu/well). After 36 h, NGF was withdrawn by washing the neurons with medium lacking NGF and adding NGF-free medium containing a neutralizing anti-NGF antibody. Two days later, neurons were fixed, stained, and counted to score for survival. To investigate localization of phospho-c-Jun, NGF withdrawal was conducted for 12 h (8).

**Immunocytochemistry and Cell Counting**—Immunocytochemistry was performed using previously published protocols (24). Briefly, cells were fixed with 3% paraformaldehyde in PBS and after blocking with 1% bovine serum albumin and 0.1% saponin in PBS, cells were probed with the M2 anti-FLAG monoclonal antibody (Eastman Kodak) followed by goat anti-mouse antibody conjugated to Alexa 488 (Jackson Immunoresearch) to visualize JBD or an anti-phospho-Ser-63 c-Jun monoclonal antibody KM-1 (Santa Cruz Biotechnology) and probed with the M2 anti-FLAG monoclonal antibody, the anti-NGF antibody for 6 h. One set of Ad-JBD-transfected neurons were deprived of NGF in a medium containing 100 μM BAF. Proteins were extracted and analyzed by Western blot using antibodies against FLAG (top panel) or GFP (second panel). The membrane was stripped and reprobed with an antibody against phospho-c-Jun (Ser-63) (third panel) and then stripped again and probed with an anti-α-tubulin antibody to demonstrate equal protein-loading efficiency (bottom panel). The same results were obtained in an independent experiment.

**RESULTS**

Newly isolated neurons were infected 4 h after plating with either Ad-FLAG-JBD or Ad-EGFP vectors at titers that gave 80–90% expression in SCG neurons after 36 h of growth in the presence of NGF (23). Neurons were then deprived of NGF. After 6 h, the cells were harvested, and the levels of expression of FLAG-JBD or EGFP as well as c-Jun phosphorylation were analyzed by Western blotting and compared with those induced in uninfected neurons or infected neurons maintained in the presence of NGF. Fig. 1 shows that NGF deprivation induced a substantial increase in c-Jun phosphorylation within 6 h of NGF deprivation in uninfected neurons, as previously described (8), as well as neurons infected with Ad-EGFP. However, infection with Ad-FLAG-JBD abrogated c-Jun phosphorylation by >90%. Equal expression of FLAG-JBD or EGFP in NGF-maintained and NGF-deprived neurons was confirmed using anti-FLAG and anti-GFP antibodies. The effect of JBD was not hampered or augmented by the presence of the pan-caspase inhibitor BAF, allowing us to compare the pattern of immunostaining of NGF-deprived neurons without losing apoptotic neurons from the coverslip.

To investigate whether JBD prevented the death of NGF-deprived neurons, neurons were infected with the appropriate Ad vectors for 36 h as above and then left to incubate for 24 h in the absence of NGF. Neurons were then fixed, stained for the presence of the FLAG epitope, and scored for the presence of apoptotic neurons from the coverslip. MTT Assays—The metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product. Absorbance was read at 550 nm on a scanning multwell spectrophotometer (Bio-Rad) after agitating the plates for 5 min on a shaker.

Western Blot Analysis—SCG neurons cultured in 12-well plates were washed three times with cold PBS and collected by centrifugation, and proteins were extracted in lysis buffer (24), separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with the M2 anti-FLAG monoclonal antibody, the anti-phospho-c-Jun monoclonal antibody KM-1 (Santa Cruz Biotechnology) or an anti-EGFP antibody (CLONTECH). Blots were then processed using the appropriate HRP-linked secondary antibody (Jackson Immunoresearch) and visualized using enhanced chemiluminescence.

**FIG. 1. Overexpression of JBD inhibits Ser-63 phosphorylation of c-Jun in NGF-deprived sympathetic neurons.** SCG neurons were infected with Ad-FLAG-JBD (Ad-JBD) or Ad-EGFP (Ad-EGFP) or left untransfected (Wt). After 36 h, neurons were maintained in medium containing NGF or were transferred to NGF-free medium containing anti-NGF antibody for 6 h. One set of Ad-JBD-transfected neurons were deprived of NGF in a medium containing 100 μM BAF. Proteins were extracted and analyzed by Western blot using antibodies against FLAG (top panel) or GFP (second panel). The membrane was stripped and reprobed with an antibody against phospho-c-Jun (Ser-63) (third panel) and then stripped again and probed with an anti-α-tubulin antibody to demonstrate equal protein-loading efficiency (bottom panel). The same results were obtained in an independent experiment.
NGF produced similar amounts of formazan after 48 h as those measured at the start of NGF deprivation.

To examine whether (i) phosphorylated c-Jun within sympathetic neurons persisted to the time when neurons would have commenced apoptotic death, and (ii) the FLAG-zJBD preserved the suppression of phospho-c-Jun to this time, neurons were treated as described above but then left to incubate in the absence of NGF for 12 h, just before apoptotic nuclear fragmentation began to appear in most neurons (Fig. 3). Very low level phospho-c-Jun staining (red) was detected in the nuclei (blue) of neurons maintained in the presence of NGF (+N) in uninfected neurons and in neurons expressing EGFP. In contrast, withdrawal of NGF in both uninfected and EGFP-expressing neurons led to a dramatic increase in phospho-c-Jun staining that was almost entirely nuclear (see purple color of nuclei in merged image). Expression of FLAG-JBD not only prevented this rise in nuclear phospho-c-Jun staining but also appeared to suppress the basal phosphorylation found in the nuclei of neurons maintained in the presence of NGF (note absence of purple-colored nuclei in merged image). Interestingly, most of the FLAG-JBD was expressed inside the cortical rim of the membrane in the neuronal cell body and was highly enriched in the growth cones (see also Fig. 2A), although diffuse staining was also observed throughout the cytoplasm. Very little expression of FLAG-JBD was observed in the nuclei (note lack of yellowish tinge in nuclei of merged image compared with nuclei of EGFP-expressing neurons).

**DISCUSSION**

The withdrawal of NGF from sympathetic neurons has previously been shown to lead to higher levels of the transcription factor c-Jun (10, 11), and it has been hypothesized that the activation of this pathway is fundamental to the process of apoptotic cell death. c-Jun levels and transcriptional activity are regulated by a number of Jun N-terminal kinases that are
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themselves regulated by MAP kinase kinases such as MKK7. As a consequence of c-Jun regulation by JNKs, a number of studies have attempted to confirm the role of JNK in mediating apoptotic death by either showing that activation of JNKs by expression of upstream kinases leads to apoptotic cell death (9, 13) or preventing the phosphorylation of JNK using a pharmacological inhibitor of MLKs, which inhibits cell death (23). However, JNK activity has not been inhibited directly in an NGF deprivation model. To investigate the role JNKs play in mediating apoptosis in sympathetic neurons, we used an adenovirus expressing the JBD of JIP-1. JIP-1 was recently identified as a scaffold protein that selectively binds intermediate kinases upstream of JNK, in addition to the JNK kinase itself (20). In this manner, it is believed that JIP-1 functions to facilitate signal transduction mediated by the bound proteins. Considering this ability to bind other kinases involved in the JNK cascade, we used a fragment of the JIP-1 molecule (residues 127–281), which encompasses a binding domain (JBD) that interacts with JNK-1, 2, and 3 but is not involved in the binding of JIP-1 to upstream effectors such as MKK7, DLK, MLK3, and HPK1 (19, 20). A recent study in cortical neurons has shown that transfection with a JBD construct prevents apoptotic death induced by arsenite (mediated via JNK3) but has shown that transfection with a JBD construct prevents apoptotic death induced by arsenite (mediated via JNK3) but no evidence was presented for suppression of JNK activity or c-Jun phosphorylation in neurons expressing JBD (12). We show here that expression of JBD is sufficient to prevent apoptotic death of NGF-deprived SCG neurons and that in doing so it abrogates the expression of phospho-c-Jun, consistent with its predicted role as the mediator of JNK-dependent apoptosis. Moreover, JBD expression also preserved much higher MTT-sensitized activity compared with Ad-0-infected neurons, consistent with the possibility that JBD prevents release of cytochrome c from the mitochondria and loss of mitochondrial function, both of which are induced by NGF deprivation (26–28). Embryonic fibroblasts from mice with targeted disruption of jnk-1 and jnk-2 are resistant to UV-induced death and are deficient in the mitochondrial death signaling pathway, including the release of cytochrome c. Thus, neurons are similar to MEFs (29) in this regard even though neurons, but not MEFs, require a transcriptional event downstream of JNK to mediate apoptotic death. Perhaps JNK signals have two death functions in the neurons, one of which acts to sensitize the mitochondria to death stimuli, with the other requiring c-Jun to complete the apoptotic process.

Although JIP is expressed as a JBD fragment, its pattern of localization appears to be similar to that reported for full-length endogenous JIP-1 and JIP-2 (19). Moreover, JBD appears to be enriched in the leading edges of growth cones (Fig. 2). Thus, although it was postulated that JIP interacts with Rho-GEF, which is enriched in growth cones, via its PTB domain (21), it is clear that the PTB domain is not necessary for growth cone localization. Whereas JBD expression is enriched under the membranes, there is also some cytoplasmic JBD but no JBD detected in the nucleus. If JBD sequesters JNK in the cytoplasm, how then might it prevent phosphorylation of c-Jun? In cerebellar granule neurons, endogenous JIP-1 was found in the nucleus where its presence was correlated with decreased c-Jun phosphorylation (30). It is possible that the antibody is simply not sensitive enough to detect nuclear JBD or that c-Jun emerges into the cytoplasm for de-phosphorylation and is tethered there by JNK. It will be necessary to analyze the neurons more carefully to resolve these questions. Thus our data are consistent with the proposal that JNK mediates a central role in SCG neuron apoptosis and that part of this effect is mediated via c-Jun phosphorylation. We propose in addition that the targets of JNK and/or c-Jun lie upstream of MEK.

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