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Insulin-like growth factor-I and Bcl-X_L inhibit c-jun N-terminal kinase activation and rescue Schwann cells from apoptosis

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Abstract

We previously reported that Schwann cells undergo apoptosis after serum withdrawal. Insulin-like growth factor-I, via phosphatidylinositol-3 kinase, inhibits caspase activation and rescues Schwann cells from serum withdrawal-induced apoptosis. In this study, we examined the role of c-jun N-terminal protein kinase (JNK) in Schwann cell apoptosis induced by serum withdrawal. Activation of both JNK1 and JNK2 was detected 1 h after serum withdrawal with the maximal level detected at 2 h. A dominant negative JNK mutant, JNK (APF), blocked JNK activation induced by serum withdrawal and Schwann cell apoptosis, suggesting JNK activation participates in Schwann cell apoptosis. Serum withdrawal-induced JNK activity was caspase dependent and inhibited by a caspase 3 inhibitor, Ac-DEVD-CHO. Because insulin-like growth factor-I and Bcl-X_L are both Schwann cell survival factors, we tested their effects on JNK activation during apoptosis. Insulin-like growth factor-I treatment decreased both JNK1 and JNK2 activity induced by serum withdrawal. LY294002, a phosphatidylinositol-3 kinase inhibitor, blocked insulin-like growth factor-I inhibition on JNK activation, suggesting that phosphatidylinositol-3 kinase mediates the effects of insulin-like growth factor-I. Overexpression of Bcl-X_L also resulted in less Schwann cell death and inhibition of JNK activation after serum withdrawal. Collectively, these results suggest JNK activation is involved in Schwann cell apoptosis induced by serum withdrawal. Insulin-like growth factor-I and Bcl family proteins rescue Schwann cells, at least in part, by inhibition of JNK activity.

Keywords: apoptosis, Bcl-X_L, caspase, c-jun N-terminal protein kinase, insulin-like growth factor-I, Schwann cells.

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Regulation of cell number by programmed cell death or apoptosis is essential during nervous system development. In the PNS, Schwann cells derived from neural crest migrate along nerves and concurrently proliferate. In rats, Schwann cell number reaches a plateau before birth and decreases while cells undergo differentiation that precedes myelin sheath formation. Recent studies show that Schwann cells undergo apoptosis in order to reach the ideal number for myelination (Stewart *et al*. 1996; Syroid *et al*. 1996, 1999 Delaney *et al*. 1999). However, the biochemical and molecular mechanisms of this process are still unclear.

C-jun N-terminal kinases (JNKs) are activated by stressful stimuli, including ultraviolet radiation, cytotoxic drugs, cytokines and other environmental stressors (Derijard *et al*. 1994; Kyriakis *et al*. 1994; Lee *et al*. 1994). JNK activation can play a role in cell proliferation, transformation, differentiation and apoptosis (Bost *et al*. 1997; Dong *et al*. 1998; Eilers *et al*. 1998). There are 10 identified isoforms of JNK originating from three homologous genes (JNK1, JNK2, and JNK3) with molecular masses of 46 or 54 kDa due to alternative splicing (Kyriakis *et al*. 1995; Gupta *et al*. 1996). JNK activation regulates gene expression by phosphorylating transcriptional factors including c-jun, Elk-1 and ATF-2 (Woodgett *et al*. 1995; Gupta *et al*. 1996; Su and Karin 1996; De Cesaris *et al*. 1999).

JNK activation mediates apoptosis in a variety of cell types. Differentiated PC12 cells undergo apoptosis when deprived of nerve growth factor (NGF) (Rukenstein *et al*. 1991). In this paradigm, NGF withdrawal correlates with

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Abbreviations used: DM, defined media; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; GST, glutathione *S*-transferase; IGF-I, insulin-like growth factor-I; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein; NGF, nerve growth factor; PtdIns, phosphatidylinositol; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

increased JNK and p38 kinase activity (Xia *et al*. 1995). Like PC12 cells, JNK activation is important in the death of sympathetic neurons (Ham *et al*. 1995), T lymphocytes (Chen *et al*. 1996) and several cancer cell lines (Pyne *et al*. 1996; Verheij *et al*. 1996; Brenner *et al*. 1997).

Insulin-like growth factor-I (IGF-I) protects neurons and Schwann cells from apoptosis. *In vitro*, IGF-I is a neurotrophic factor for sensory, sympathetic and motor neurons (Feldman *et al*. 1997; Russell *et al*. 1998; Leventhal *et al*. 1999). We have shown that osmotic stress induces apoptosis in neurons and that IGF-I, but not NGF, epidermal growth factor (EGF) or fibroblast growth factor (FGF), serves as a neuroprotectant (Matthews and Feldman 1996; Singleton *et al*. 1996b; Matthews *et al*. 1997; van Golen *et al*. 2000; Russell *et al*. 1999; van Golen and Feldman 2000). IGF-I binds to the cell surface IGF-I receptor and activates two main downstream pathways: the phosphatidylinositol (PtdIns)-3 kinase and mitogen activated protein (MAP) kinase pathways. We reported that IGF-I rescues neuronal cells (Singleton *et al.* 1996b; van Golen and Feldman 2000) and Schwann cells (Delaney *et al.* 1999) from apoptosis via the PtdIns-3 kinase pathway. In parallel, we find that IGF-I inhibits high glucose-induced apoptosis and JNK activation in neuroblastoma cells, suggesting that inhibition of JNK activation may mediate IGF-I neuroprotection (Cheng and Feldman 1998). Our ideas are supported by two recent reports: one from Campana *et al*. (1999) that IGF-I blocks Schwann cell apoptosis via PtdIns-3 kinase, and a second report from Okubo *et al*. (1998) that IGF-I prevents the death of 293 kidney cells by blocking JNK activity via a PtdIns-3 kinase pathway.

The Bcl family of proteins contains both anti-apoptotic (Bcl-2 and Bcl-X_L), and pro-apoptotic (Bad and Bax) (Davies 1995; Adams and Cory 1998) family members. Bcl-2 and Bcl-X_L are mitochondrial membrane proteins that regulate membrane polarity. In response to apoptotic insults, mitochondria are depolarized and release cytochrome *c* which in turn triggers cytoplasmic caspase activity and induces apoptosis. Bcl-2 and Bcl-X_L dimerize and form channels in mitochondrial membranes to stabilize the membrane potential and prevent cytochrome *c* release (Kharbanda *et al*. 1997; Kluck *et al*. 1997). In contrast, Bad and Bax bind to Bcl-2 and Bcl-X_L to interrupt Bcl channel formation (Davies 1995; Adams and Cory 1998). Collectively, the balance between pre-apoptotic and anti-apoptotic Bcl family proteins determines the susceptibility of a cell to apoptosis.

In this study, we examined the role of JNK in Schwann cell apoptosis. Serum withdrawal mediates Schwann cell apoptosis and in parallel activates JNK. Dominant negative JNK (APF) partly rescues Schwann cells from serum withdrawal apoptosis, suggesting that JNK activation contributes to Schwann cell apoptosis induced by removing serum. Serum withdrawal-induced JNK activity is caspase dependent and inhibited by the caspase 3 inhibitor, Ac-DEVD-CHO. Both IGF-I treatment and overexpression of Bcl-X_L block JNK activation in Schwann cells and, in parallel, rescue Schwann cells from serum withdrawal apoptosis. Collectively, these data suggest a pivotal role for JNK in Schwann cell apoptosis and imply that IGF-I and Bcl-X_L, may, in part, protect Schwann cells from cell death by regulating JNK activity.

Materials and methods

Recombinant human IGF-I was a generous gift of Cephalon (West Chester, PA, USA) and was stored in 100 mM acetic acid at −80 °C. JNK1 (FL), NK2 (N18) and HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). JNK1 (FL) recognizes JNK1 p46, JNK2 p54, and JNK3 p54. JNK2 (N18) is more specifically reactive to JNK2 and, to a lesser extent, JNK1 and JNK3. All media, sera and bovine pituitary extract were from Gibco (Grand Island, NY, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

Schwann cells were isolated from the sciatic nerves of 3-day-old Sprague-Dawley rats (Harlan-Sprague Dawley, Indianapolis, IN, USA) as described previously (Brockes *et al*. 1979). Schwann cells were cultured on poly(L-lysine)-coated plates in culture media [Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum, 2 μM forskolin and 10 mg/mL bovine pituitary extract]. Cells were passaged upon confluency and used for 4 passages. For serum-free conditions, Schwann cells were washed twice and cultured in defined media (DM) [DMEM/F12 media supplemented with transferrin (10 mg/L), putresine (10 mM), progesterone (20 nM), sodium selenite (30 nM)]. For experiments using kinase or caspase inhibitors (PD98059, LY294002 and Ac-DEVD-CHO), cells were pretreated with inhibitors with indicated concentrations in serum-free conditions 30 min prior to IGF-I administration.

CDNA constructs

HA-tagged JNK1 cDNA was a generous gift from Dr Derek Leroith (National Institutes of Health, Bethesda, MD, USA). The dominant negative JNK (APF) construct was kindly given by Dr Roger Davis (Howard Hughes Medical Institute, University of Massachusetts Medical Center, Worcester, MA, USA). JNK (APF), in which the phosphorylation site Thr-Pro-Tyr is changed to Ala-Pro-Phe, behaves as a dominant negative mutant and blocks JNK signaling (Derijard *et al.* 1994; Zeigler *et al*. 1999). Wild-type Bcl-X_L cDNA was provided by Dr Valerie Castle (University of Michigan). All the cDNAs were inserted into pcDNA 3.1 expression vectors.

Cell transfection

Schwann cells were transfected using lipofectamine (Singleton *et al*. 1996a). Following the manufacturer's protocol, 8 μg of cDNA was incubated with 30 μL of lipofectamine for 45 min before adding to cells cultured in 100-mm tissue culture plates in DM. After 6 h, DM was replaced by culture media for overnight recovery then selected by 425 μg/mL G418 in culture media for at least 4 weeks before use.

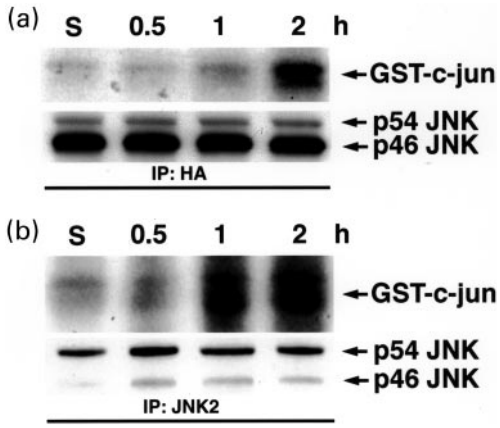

Fig. 1 JNK1 is activated after serum withdrawal. (a) HA-JNK1 is activated after serum withdrawal. Schwann cells overexpressing HA-JNK1 were either treated with serum (S) or serum deprived for 0.5–2 h. HA-JNK1 was immunoprecipitated (IP) by a HA antibody. The JNK activity assay was performed and GST-c-jun phosphorylation was detected at 1 h after serum withdrawal with maximal effects detected at 2 h. Level of total JNK1 loaded was determined by JNK1 (FL) immunoblots. (b) Serum withdrawal activates JNK2. Schwann cells were either treated with serum (S) or were serum deprived for 0.5–2 h. JNK2 was immunoprecipitated by an antibody specific to JNK2 followed by the JNK activity assay. JNK2 is activated after 1 h of serum withdrawal and the activity increases up to 2 h. The level of total JNK2 loaded was determined by JNK2 (N18) immunoblots. Data are from one of three representative experiments.

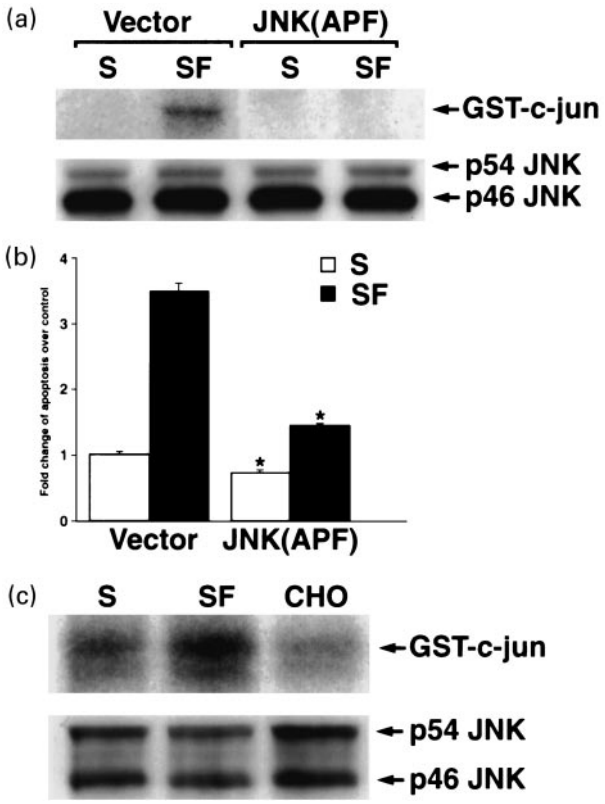

Fig. 2 Dominant negative JNK mutant inhibits JNK activation and apoptosis induced by serum withdrawal. (a) Schwann cells transfected with control vector or JNK (APF) were in 10% serum (S) or serum-free (SF) media for 2 h. JNK1 immunoprecipitation was performed followed by the JNK kinase assay. The JNK activation induced by serum withdrawal is inhibited by a dominant negative JNK mutant, JNK (APF) (upper). The level of total JNK1 loaded was determined by JNK1 (FL) immunoblots (lower). (b) Apoptosis of Schwann cells after 24 h of serum withdrawal was measured by flow cytometry. Serum withdrawal enhanced Schwann cell apoptosis 3.5-fold over the serum control (in which baseline apoptosis is 8%) in control vector transfected Schwann cells. In contrast, Schwann cells expressing JNK (APF) have significantly less cell death than the control vector transfected Schwann cells after serum withdrawal. Data are expressed as mean fold change over serum treated control ± SEM. $^*p < 0.05$ in comparison with control vector transfected cells treated with the same condition by unpaired two-tailed *t*-test using data from three independent experiments. (c) Caspase inhibitor blocks JNK activation. Untransfected Schwann cells were incubated in serum (S) or serum-free (SF) conditions for 2 h ± 2 μg/mL Ac-DEVD-CHO (CHO), a caspase 3 inhibitor. A JNK1 activity assay was performed using JNK1 (FL) for immunoprecipitation. CHO significantly blocks JNK activation. The level of total JNK1 loaded was determined by JNK1 (FL) immunoblots. Data are from one of three representative experiments.

Immunoblotting

Whole-cell lysates were collected using lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP 40, 150 mM NaCl, 1 mM EDTA, 1 mM $PhCH_2SO_2F$, 1 μg/mL aprotinin and leupeptin, 1 mM sodium orthovanadate, and 1 mM NaF). Fifty micrograms of protein was separated by sodium dodecyl sulfate (SDS)–PAGE in 15% polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked, and incubated with primary antibodies against HA, JNK1 (FL) or JNK2 (N18) (1 : 1000) (Cheng and Feldman 1998). Immunoreactive proteins were identified by horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence reagents from Amersham (Arlington Heights, IL, USA).

JNK activity assay

Three hundred micrograms of protein from each sample was incubated with 4 μg/mL anti-HA, anti-JNK1 (FL) or JNK2 (N18) antisera overnight at 4°C and precipitated by protein A/G agarose beads (Santa Cruz). The immunoprecipitants were rinsed twice with kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 0.1 mM Na_3Vo_4, 10 mM $MgCl_2$) and resuspended in 30 μL of kinase buffer containing 2 μg glutathione *S*-transferase (GST) c-jun (New England Biolabs, Beverly, MA, USA), 100 μM ATP and 10 μCi [γ-^{32}P] ATP (3000 Ci/mmol, Amersham) and incubated for 30 min at 30°C. The kinase reaction was terminated by the addition of Laemmli sample buffer and boiled for 5 min. Samples were resolved in a 15% polyacrylamide gel, transferred to a nitrocellulose membrane and evaluated by autoradiography. To determine the equality of sample loading, membranes were also processed for JNK1 (FL), JNK2 (N18) or HA immunoblotting procedures.

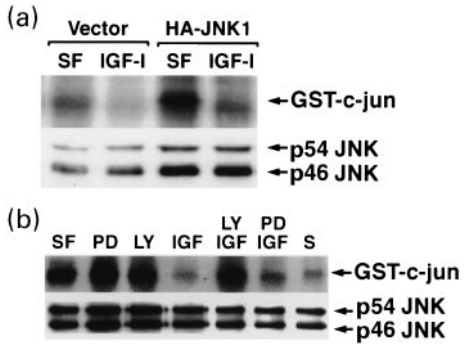

Fig. 3 IGF-I inhibits JNK activity via PtdIns-3 kinase. (a) IGF-I inhibits JNK activation induced by serum withdrawal. Schwann cells were serum deprived for 2 h ± 10 nM IGF-I. A JNK activity assay was performed following JNK immunoprecipitation. In both control vector and HA-JNK1 transfected Schwann cells, serum deprivation activates JNK. In both cases, IGF-I treatment decreases JNK activity. (b) In untransfected Schwann cells, IGF-I inhibition of JNK activity is blocked by LY294002 but not PD98059. Schwann cells were incubated in 10% serum (S) or serum-free (SF) conditions for 2 h ± 10 nM IGF-I and/or 10 μM PD98059 (PD) and 10 μM LY294002 (LY). In conditions with PD and LY, Schwann cells were pretreated with the indicated inhibitor for 30 min before serum withdrawal and IGF-I treatment. JNK activity assays demonstrate that LY294002 blocks IGF-I inhibition on JNK activity. Neither inhibitor has an effect on JNK activation induced by serum withdrawal. Data are from one of three representative experiments.

Flow cytometry

Schwann cells were serum deprived in DM ± 10 nM IGF-I for 24 h and processed as described previously (Delaney *et al.* 1999). Flow cytometry data were collected from an Epics Elite flow cytometry system with cells in sub-G_0 phase considered apoptotic.

Results

Serum withdrawal increases JNK activity in Schwann cells

To study the roles of JNK1 in Schwann cell apoptosis induced by serum withdrawal, Schwann cells were transfected with HA-JNK1. The level of both p46 and p54 kDa JNK1 expression increased markedly after HA-JNK1 overexpression in comparison with the control vector transfected cells (data not shown). The 46 kDa isoform is the predominant expression product in both control and HA-JNK1 overexpressors.

To test whether serum withdrawal activates JNK1, Schwann cells overexpressing HA-JNK1 were placed in either media containing 10% fetal bovine serum or in serum-free conditions for 0.5–2 h. To isolate the HA-JNK1 from cell lysates, 300 μg of protein was immunoprecipitated using an anti-HA antibody. The immunoprecipitates were then processed for an *in vitro* JNK kinase assay using GST-c-jun as a substrate. The levels of GST-c-jun phosphorylation visualized by autoradiography represents JNK activity. HA-JNK1 was activated after Schwann cells were serum deprived for 1 h with more activity detected after 2 h in the serum-free condition (Fig. 1a). To determine whether serum withdrawal also activates JNK2, Schwann cells were treated with the same experimental paradigm and JNK2 activity was measured by the JNK activity assay following JNK2 immunoprecipitation (Fig. 1b).

JNK mediates Schwann cell apoptosis in a caspase-dependent manner

The role of JNK activation in Schwann cell apoptosis was studied using a dominant negative mutant of JNK, JNK (APF). Schwann cells overexpressing JNK (APF) and Schwann cells transfected with control vector were serum deprived for 2 h and processed for JNK kinase activity. JNK (APF) expression completely blocked JNK activation induced by serum withdrawal (Fig. 2a). Cell death studies using flow cytometry demonstrated that serum withdrawal induced Schwann cell apoptosis in control vector transfected cells up to 3.5-fold over that observed in control vector transfected Schwann cells maintained in serum (Fig. 2b). In contrast, removing serum resulted in reduced levels of apoptosis in Schwann cells transfected with JNK (APF), suggesting that JNK activation plays an active role in Schwann cell apoptosis (Fig. 2b).

We previously reported that Schwann cells undergo caspase-dependent apoptosis upon removal of serum and that pretreatment of Schwann cells with caspase inhibitors blocks serum withdrawal apoptosis (Delaney *et al.* 1999). In this study, we examined the relationship between caspase activity and JNK activation. Schwann cells were pretreated with 2 μg/mL of the caspase 3 inhibitor Ac-DEVD-CHO (CHO) prior to placing the Schwann cells in serum-free media. JNK activity was measured using the JNK kinase activity assay after 2 h. Pretreatment with Ac-DEVD-CHO significantly inhibits JNK activation (Fig. 2c). These data suggest that JNK activation is downstream of the caspase cascade and that caspase activity is required for activation of JNK in the serum withdrawal paradigm.

IGF-I blocks JNK activation and Schwann cell apoptosis via PtdIns-3 kinase signaling

We previously reported that IGF-I blocks Schwann cell apoptosis upon serum withdrawal via activation of PtdIns-3 kinase (Delaney *et al.* 1999). In this study, we examined the relationship between the anti-apoptotic effects of IGF-I and JNK activity. We speculated that IGF-I downstream signaling of PtdIns-3 kinase would inhibit JNK. To test our hypothesis, Schwann cells transfected with control vector or HA-JNK1 were serum deprived for 2 h ± 10 nM IGF-I. Total JNK (endogenous JNK1 and JNK2 plus transfected HA-JNK1) was assessed by immunoprecipitation using a JNK1 (FL) antibody that recognizes both JNK1 and JNK2. As a result of a higher level of HA-JNK1

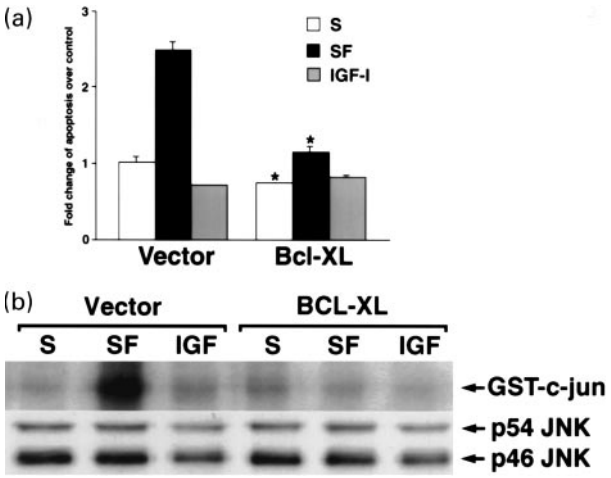

Fig. 4 Bcl-X_L overexpression rescues Schwann cells and inhibits JNK activation. (a) Bcl-X_L overexpression rescues Schwann cells from apoptosis. Schwann cells transfected with Bcl-X_L or control vector were serum deprived for 24 h ± 10 nM IGF-I and then processed for flow cytometry analysis to detect apoptosis. Bcl-X_L overexpression effectively reduced Schwann cell apoptosis in serum (S) and serum-free (SF) conditions. In both control vector and Bcl-X_L transfected Schwann cells, IGF-I rescues Schwann cells from apoptosis. Data are expressed as mean fold change over serum treated vector control ± SEM. *$p < 0.05$ in comparison with control vector transfected cells treated with the same condition by unpaired two-tailed *t*-test using data from three independent experiments. (b) Bcl-X_L overexpression inhibits JNK activation induced by serum withdrawal. Schwann cells transfected with control vector or Bcl-X_L were incubated under 10% serum (S) or serum-free (SF) conditions for 2 h ± 10 nM IGF-I. JNK activation induced by serum withdrawal is inhibited by Bcl-X_L overexpression. Data are from one of three representative experiments.

expression, more JNK activity was detected after serum withdrawal in Schwann cells overexpressing HA-JNK1 than the control Schwann cells (Fig. 3a). IGF-I treatment consistently inhibited JNK activation in control vector and HA-JNK1 transfected cells (Fig. 3a).

IGF-I activates two main downstream pathways, the MAP kinase and PtdIns-3 kinase pathways. To determine which of these two pathways was instrumental in JNK regulation, Schwann cells were treated with pathway inhibitors for MAP kinase (PD98059) and PtdIns-3 kinase (LY294002). Schwann cells were serum deprived for 2 h ± 10 nM IGF-I. Ten micromolar PD98059 or 10 μM LY294002 was applied 30 min prior to serum withdrawal and also co-administered with IGF-I. JNK kinase activity was measured after 2 h. Two hours of serum withdrawal strongly enhanced JNK activity that was blocked by IGF-I treatment (Fig. 3b). LY294002 (LY) but not PD98059 (PD) decreased IGF-I inhibition of JNK activity, suggesting that the PtdIns-3 kinase pathway primarily mediates IGF-I inhibition of JNK activity. Both inhibitors have no effect on JNK activation induced by serum withdrawal (Fig. 3b).

Bcl-X_L overexpression blocks Schwann cell apoptosis and JNK activity

Similar to IGF-I, Bcl-X_L overexpression has been shown to rescue a variety of cells from apoptosis (Parsadanian *et al*. 1998; Vander Heiden *et al*. 1999; Xu *et al* 1999). To establish the role of JNK as a common regulator for both growth factor (IGF-I) and nongrowth factor (Bcl-X_L) mediated rescue, we overexpressed Bcl-X_L in Schwann cells. Schwann cells transfected with Bcl-X_L express significantly higher levels of Bcl-X_L protein than control vector transfected cells (data not shown). To test if Bcl-X_L overexpression rescues Schwann cells, both control vector or Bcl-X_L transfected Schwann cells were treated in 10% fetal bovine serum (S), serum-free (SF), or SF + 10 nM IGF-I for 24 h. Cell death studies revealed that, similar to IGF-I, Bcl-X_L overexpression effectively reduced Schwann cell apoptosis induced by serum withdrawal (Fig. 4a). However, in contrast to IGF-I, the effect of Bcl-X_L overexpression is partial (compare S with SF). Further addition of IGF-I in Bcl-X_L transfected cells resulted in a complete rescue (Fig. 4a). Besides rescuing Schwann cells from apoptosis, Bcl-X_L overexpression also inhibited JNK activation after 2 h of serum withdrawal (Fig. 4b).

Discussion

JNK is activated by a variety of apoptotic stressors. However, the role of JNK in apoptosis is still under debate. In this study, serum withdrawal mediates Schwann cell apoptosis and JNK activation. After 2 h, serum removal activates JNK and within 6 h Schwann cells display the morphologic and biochemical changes associated with apoptosis (Delaney *et al.* 1999). Our results suggest that JNK is instrumental in Schwann cell apoptosis. In agreement with our findings, following NGF withdrawal, JNK and P38 kinase are activated in PC12 cells preceding apoptosis (Xia *et al.* 1995) and in human B lymphocytes, JNK activation mediates IgM-induced apoptosis (Graves *et al*. 1996). JNK activation is also required in ceramide-mediated apoptosis (Verheij *et al.* 1996) and, in several cell types, activation of either the upstream kinase of JNK (ASK1) or downstream c-jun activity contributes to apoptosis (Ichijo *et al*. 1997). When JNK activation was blocked in our studies by overexpression of JNK (APF), Schwann cells underwent substantially less apoptosis upon serum withdrawal. Similar findings are reported in ovarian cancer cells where dominant negative JNK1 significantly reduces paclitaxel-mediated cell death (Lee *et al*. 1998).

The correlation between JNK activation and apoptosis appears to be cell and stressor specific. While our results suggest that JNK activation is instrumental in Schwann cell

apoptosis, in other cell types JNK activation promotes cell survival. For example, in breast cancer cells, JNK activation is induced by tumor necrosis factor (TNF) yet neither dominant negative MAP kinase kinase 4 (MKK4), nor c-jun have effects on TNF-mediated apoptosis (Ameyar *et al.* 1998). These data suggest that the cell death that occurs in response to TNF is unrelated to JNK activation. In parallel, Chauhan *et al.* (1997) reported that dexamethasone-induced apoptosis of multiple myeloma cells is also a JNK-independent event. Park *et al.* (1996) also describe JNK-dependent and JNK-independent rescue of PC12 cells from apoptosis induced by serum withdrawal and their conclusions are supported by studies from Wang *et al.* (1999). Several reports suggest that JNK activation may actually promote cell survival. Potapova *et al.* (1997) found that JNK activation is required for DNA repair and protects glioblastoma cells from cisplatin toxicity. In HeLa cells, expression of dominant negative JNK enhances apoptosis induced by photodynamic therapy with hypericin (Assefa *et al.* 1999). When comparing these published reports with this study, one clear difference arises: our data are based on work in primary nontransformed cells while other reports rely on cancer and transformed cell lines. Collectively, these results imply a potential difference in the regulation of cell death pathways by JNK between primary and neoplastic cells and reinforce the concept that inhibition of JNK activity is not a universal mechanism for cell survival.

In this study, serum withdrawal-induced JNK activation in Schwann cells is caspase dependent. Similar findings are reported in other cell types, including prostate cancer cells (Chen *et al.* 1999), epidermal keratinocytes (Sanchez-Perez & Perona 1999), and Jurkat T lymphocytes (Srivastava *et al.* 1999). The mechanism for caspase mediated JNK activation has been studied by Chaudhary and colleagues. They provide evidence that caspase 8, an upstream activator of caspase 3, directly interacts with the TNF receptor via the receptor's death effector domain and activates MAP or Erk kinase kinase 1 (MEKK1), an upstream kinase of the JNK pathway (Chaudhary *et al.* 1999).

In this study, IGF-I blocked the increase in JNK activity that occurs upon removal of serum in Schwann cells. In this same paradigm, IGF-I blocks Schwann cell apoptosis (Delaney *et al.* 1999). Our results agree with those of Campana *et al.* (1999) who found that IGF-I and prosaposin block serum withdrawal-mediated Schwann cell apoptosis. We previously reported that IGF-I inhibits JNK activation and apoptosis in neuroblastoma cells (Cheng and Feldman 1998; van Golen and Feldman, 2000). In agreement with our findings, Okubo *et al* (1998) report that IGF-I protects kidney 293 cells overexpressing JNK1 from apoptosis. Our results coupled with our previous reports suggest that JNK plays a vital role in apoptosis in cells of the nervous system, and that inhibition of JNK activity serves as a potential pathway for IGF-I protection. Similar to IGF-I, vascular endothelial growth factor (VEGF) prevents apoptosis of human microvascular endothelial cells by blocking JNK activation in cell death models of serum withdrawal and ceramide treatment (Gupta *et al.* 1999). In contrast, EGF may activate JNK and promote growth in selected cancer cell lines (Minden *et al.* 1994; Bost *et al.* 1997). The fact that JNK activity can be both increased and decreased in response to individual growth factors suggests distinct roles for JNK in response to these factors and their effects on multiple cell types.

Our studies show that IGF-I inhibition of JNK activity is mediated by PtdIns-3 kinase in Schwann cells. The PtdIns-3 kinase inhibitor LY294002 blocked IGF-I inhibition of JNK activity, whereas PD98059, an inhibitor of the MAP kinase pathway, had no effect. Our data agree with the findings of Okubo *et al.* (1998). In their studies, the ability of IGF-I to suppress JNK activity was blocked by both LY294002 and expression of a dominant-negative Akt construct. Our results and those of Okubo *et al.* (1998) suggest that IGF-I may block JNK activation via an IGF-I/PtdIns-3 kinase/Akt pathway (Delaney *et al.* 1999; van Golen *et al.* 2000). This pathway in turn can block activation of the caspase cascade (Delaney *et al.* 1999), an event required for JNK activity in Schwann cells. Alternatively, IGF-I-mediated JNK inhibition could occur via growth factor activation of cytoskeletal elements (Leventhal *et al.* 1997; Kim and Feldman 1998b; Kim *et al.* 1998a), the MAP kinase pathway (Cheng and Feldman 1998) or a recently discovered JNK inhibitory kinase (JIK) (Tassi *et al.* 1999). We are currently investigating the signaling mechanisms underlying IGF-I regulation of JNK activity.

Mitochondrial integrity is controlled by the Bcl-2 family proteins (Adams and Cory 1998; Kroemer *et al.* 1998; Gross *et al.* 1999). This family includes the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Usdin and Fischbach 1986; Evan and Littlewood 1998). Bcl-X_L encodes a protein that forms membrane channels in the outer mitochondrial membrane (Schendel *et al.* 1998). Bcl-X_L maintains a normal mitochondrial membrane potential (Ψ) and in turn blocks apoptosis. Loss of normal mitochondrial potential leads to the release of cytochrome *c* into the cytosol, initiating a hierarchical activation of caspases with eventual apoptosis (Vander Heiden *et al.* 1997; Slee *et al.* 1999). Schwann cells express Bcl-X_L and little Bcl-2 (unpublished data). Our data demonstrate that Bcl-X_L protects Schwann cells from apoptosis and blocks JNK activation. In support of our findings, Pandey *et al.* (1999) report that Bcl-X_L overexpression inhibits methylmethane sulfonate induced JNK activation, whereas Park *et al.* (1997) found that overexpression of Bcl-2 inhibits JNK activation in N18TG cells. Both Bcl-2 and Bcl-X_L overexpression inhibit JNK activation in Jurkat T cells (Chen *et al.* 1998) and Bcl-2 overexpression in Schwann cells blocks serum withdrawal apoptosis (Soilu-Hanninen *et al.* 1999). The mechanism

whereby Bcl proteins block JNK activation is under investigation. One unifying concept lies in the well-established ability of anti-apoptotic Bcl family members to block activation of the caspase cascade which in turn could block JNK activation. However, the answer may not be this straightforward. In a recent report, Pandey *et al.* (1999) discovered that the ability of Bcl-X_L to block JNK activity is dependent on phosphatase activity. Clearly, more work is needed in this area of investigation.

In summary, our data demonstrate JNK activation is instrumental in mediating apoptosis in Schwann cells following serum withdrawal. JNK appears to be an intermediate signal downstream of caspases. JNK activation is blocked by multiple anti-apoptotic factors, including IGF-I and overexpression of Bcl-X_L. We are currently investigating the caspase cascade required for JNK activity in Schwann cells and the mechanisms underlying the ability of IGF-I to block JNK activity and rescue Schwann cells from apoptosis.

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