

Inhibition of apoptosis by Nur77 through NF- κ B activity modulation

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Abstract

The orphan nuclear receptor Nur77 has been described as a mediator of apoptosis and has also been associated with growth promotion and apoptotic resistance. This study aimed at evaluating the contribution of Nur77 to different apoptotic stimuli. Nur77 overexpression in the fibroblastic cell line HEK293 promoted resistance to programmed cell death induced by death receptor engagement, DNA-damaging agents and endoplasmic reticulum stress. Nur77 overexpression led to enhanced NF- κ B activity, and DNA-binding inhibitors confirmed the contribution of NF- κ B to Nur77 antiapoptotic activity. Nur77 overexpression leads to NF- κ B-dependent induction of the antiapoptotic gene cIAP1. Paradoxically, while dominant-negative Nur77 expression sensitised cells to Fas ligand-induced cell death, it protected cells from endoplasmic reticulum stress apoptosis in a manner similar to wild-type Nur77. These results show that nuclear crosstalk between Nur77 and other transcription factors contribute to cell fate in response to different apoptosis-inducing agents.

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Abbreviations: ALLN, acetyl-leucyl-leucyl-norleucinal; CAPE, caffeic acid phenyl ester; CHX, cycloheximide; CPT, camptothecin; DN, dominant negative; ER, endoplasmic reticulum; FasL, Fas ligand; IAP, inhibitor of apoptosis; KBK, kamebakaurin; LBD, ligand-binding domain; NurRE, Nur response element; PMA, phorbol 12-myristate 13-acetate; TG, thapsigargin; VP16, etoposide

Introduction

Nur77 (NGFI-B, TR3, NR4A1) is an orphan nuclear receptor induced by various stimuli such as growth promotion and stress. It was originally identified as an immediate-early gene induced by serum growth factors in fibroblasts¹ and nerve growth factor in PC12 cells.² Nur77 is the first member of the NGFI-B family that comprise Nurr1 and NOR-1, and all

members possess an AF-1 N-terminal transactivation domain, a zinc-finger DNA-binding domain and an AF-2 C-terminal segment containing the ligand-binding domain (LBD). However, no ligands for these receptors have been identified and the LBD appears nonfunctional.³

Nur77 activity can be induced by phorbol esters, membrane depolarisation, cAMP or calcium ionophores^{4,5} and Nur77 levels are sharply upregulated in thymocytes and T-cell hybridomas upon T-cell receptor engagement.^{6,7} Nur77 can bind as a monomer on NGFI-B-responsive element sequences or as a homodimer on tandem inverted NBRE sequences.^{8,9} Nur77 also binds the Nur response element (NurRE) as a homodimer or a heterodimer with other NGFI-B family members.¹⁰ Nur77 can form heterodimers with the retinoid X receptor and bind DR5 sequences in the presence of 9-*cis*-retinoic acid to contribute to retinoid-dependent transcription.¹¹ Thus, Nur77 has the potential to transactivate several different promoter elements and its transcriptional contribution to cell fate is dependent on cellular context and the types of external signals received.

Nur77 is required for T-cell activation-induced apoptosis and thymocyte negative selection.^{6,7,12} While transgenic Nur77 overexpression leads to massive thymocyte cell death, Nur77-deficient mice show no defects in negative selection, arguing that other NGFI-B members can substitute for Nur77.¹³ Indeed, transgenic expression of a dominant negative (DN) Nur77 totally blocks thymocyte apoptosis, confirming the redundant activity of other NGFI-B family members.¹⁴ The precise mechanisms involved in Nur77-mediated apoptosis remain ill-defined but a link between transcriptional activity and apoptosis has been proposed,^{15,16} yet Nur77 nuclear export can lead to mitochondrial cytochrome *c* release and apoptosome activation.^{17,18} On the other hand, Nur77 expression can prevent ceramide-induced apoptosis in B cells and tumour necrosis factor-induced cell death in macrophages.^{19,20} Nur77 can therefore contribute to cell death or survival depending on the cell type and the nature of proapoptotic signal received.

We evaluated the contribution of Nur77 to programmed cell death triggered by different proapoptotic stimuli in a fibroblastic cell line. Cell death induced by etoposide (VP16), camptothecin (CPT), thapsigargin (TG) or low doses of Fas ligand (FasL) combined with cycloheximide (CHX) were significantly decreased in Nur77-transfected HEK293 cells. Nur77 enhanced NF- κ B activity, which promoted antiapoptotic gene expression, and NF- κ B DNA-binding inhibitors blocked the antiapoptotic activity of Nur77. However, expression of DN Nur77 enhanced FasL-induced apoptosis as expected, but had no effect on cell death induced by DNA damage and blocked ER stress apoptosis in a manner similar to wild-type Nur77. Altogether, these results show that Nur77 can modulate apoptosis through NF- κ B crosstalk and that further complexity exist depending on the nature of the apoptotic signal received.

Results

Nuclear Nur77 inhibits different apoptotic pathways

Clones expressing Nur77 were obtained by stable transfection of the fibroblastic cell line HEK293 and the transfectants had morphological and growth properties identical to parental or vector-transfected cells. Susceptibility to DNA-damaging agents and endoplasmic reticulum (ER) stress was compared between vector-transfected cells and Nur77-expressing cells showed reduced apoptosis in response to VP16 and CPT, as well as ER stress triggered by TG (Figure 1a). While Nur77-expressing cells showed similar susceptibility to death induced by FasL treatment (Figure 1b), they were highly resistant to low doses of FasL combined with CHX (Figure 1c). Since Nur77 only protected from FasL apoptosis in the presence of CHX, this suggests that short-lived antiapoptotic factors are involved in this phenomenon. These results were reproduced with another Nur77 clone and a pool of stable transfectants (Figure 2a) and apoptosis protection correlated to Nur77 levels as assessed by NurRE reporter transactivation levels (Figure 2b). Clone #5 providing maximal protection was selected for further experiments.

It has been shown that Nur77 can exert its proapoptotic activities through mitochondrial localisation and cytochrome *c* release.^{17,18} Cells transiently transfected with GFP-tagged Nur77 were subjected to proapoptotic stimuli and analysed by confocal microscopy. While PMA treatment lead to limited nuclear export as described,^{17,18} no cytoplasmic staining or mitochondrial colocalisation was observed with other treatments (data not shown). Since VP16 can cause Nur77 nuclear export in other cell types,¹⁸ this indicates that this phenomenon is not universal and nuclear Nur77 is likely involved in inhibiting HEK293 cell apoptosis.

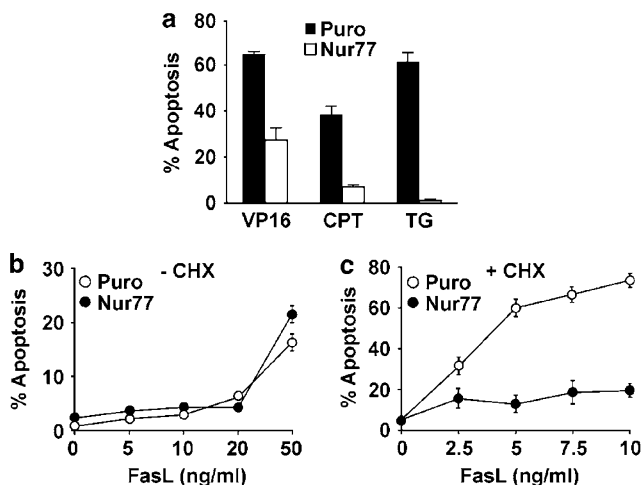


Figure 1 Nur77 inhibits different apoptotic pathways. (a) 10^5 HEK293 cells transfected with empty vector (puro) or Nur77 (Nur77) plated in 24-well culture dishes were treated for 48 h with $100 \mu\text{M}$ etoposide (VP16), $10 \mu\text{M}$ camptothecin (CPT) or $1 \mu\text{M}$ thapsigargin (TG). Results are expressed as percent-specific apoptosis relative to untreated controls. Data shown represent the mean \pm S.D. of three separate experiments. Stable transfectants were treated 24 h with various concentrations of FasL (b) or FasL and $0.5 \mu\text{g/ml}$ cycloheximide (CHX) (c). Apoptosis is expressed as the mean \pm S.D. of triplicates

Nur77 increases NF- κ B activity in a proteasome-independent manner

Several apoptotic pathways are regulated at the transcriptional level and the NF- κ B transcription factor contributes to the expression of short-lived prosurvival factor such as inhibitors of apoptosis (IAPs) and cFLIP.²¹ NF- κ B is normally sequestered in the cytoplasm by I κ B and stimuli that trigger I κ B phosphorylation lead to proteosomal degradation and NF- κ B nuclear translocation.²² Acetyl-leucyl-leucyl-norleucinal (ALLN) is a peptide aldehyde that inhibits the proteasome and prevents I κ B degradation, while caffeic acid phenyl ester (CAPE) and kamebakaurin (KBK) form covalent adducts with NF- κ B family members and prevent DNA binding, but not translocation.^{23,24} The impact of Nur77 expression on NF- κ B activity in stable transfectants was evaluated by transient transfection of an NF- κ B luciferase reporter, and Nur77 expression increased NF- κ B activity by over three-fold (Figure 3a). The contribution of the proteosomal pathway to Nur77-mediated increase in NF- κ B activity was evaluated using different inhibitors. ALLN treatment had no effect on NF- κ B activity enhancement and only DNA-binding inhibitors reduced NF- κ B activity in Nur77-transfected cells (Figure 3a). Phorbol 12-myristate 13-acetate (PMA) treatment was used as positive control to confirm inhibitor effectiveness and ALLN, CAPE and KBK all strongly reduced reporter transactivation

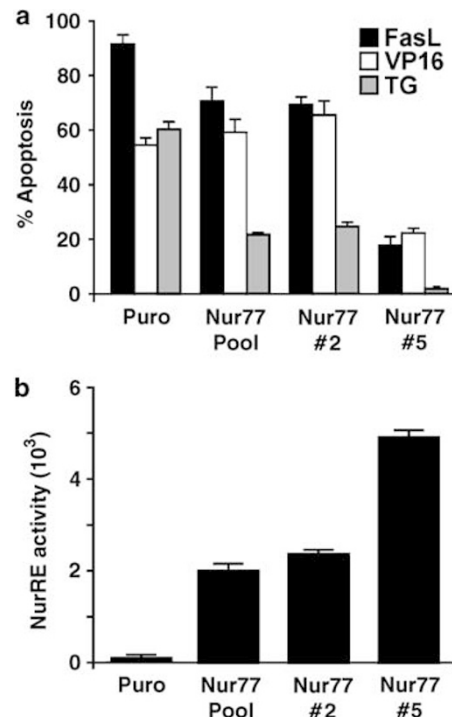


Figure 2 Nur77 levels correlate with protection from apoptosis. (a) HEK293 cells transfected with empty vector (puro) or Nur77 (pool of stable transfectants or clones #2 and #5) were plated in 24-well culture dishes and treated 48 h with 5 ng/ml of FasL and $0.5 \mu\text{g/ml}$ CHX, $100 \mu\text{M}$ etoposide (VP16) or $1 \mu\text{M}$ thapsigargin (TG). Results are expressed as the mean \pm S.D. of percentage-specific apoptosis of triplicates relative to untreated controls. (b) The same cells were seeded in triplicate in six-well dishes and transiently transfected with the NurRE-Luc reporter plasmid. Results shown are the mean \pm S.D. of NurRE activity normalised on β -galactosidase activity

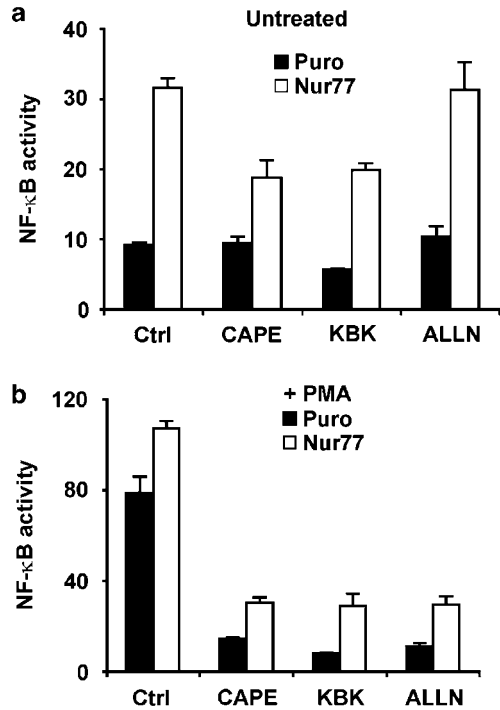


Figure 3 Nur77 expression enhances nuclear NF- κ B activity. (a) HEK293 cells transfected with empty vector (puro) or Nur77 (Nur77) were transiently transfected with an NF- κ B luciferase and β -galactosidase reporters and treated for 16 h with 100 μ M caffeic acid phenyl ester (CAPE) or 100 μ M acetyl-leucyl-leucyl-norleucinal (ALLN), or 2 h with 5 μ g/ml kamebakaurin (KBK). (b) Stable transfectants were transiently transfected with reporters and treated with NF- κ B inhibitors as in (a) and then treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 4 h before cell lysis. Data shown in both panels are the mean relative luminescence \pm S.D. of triplicates normalised on β -galactosidase activity

(Figure 3b). These results support the idea that Nur77 modulates NF- κ B activity from within the nucleus.

Nuclear NF- κ B inhibition blocks the antiapoptotic actions of Nur77

To evaluate the contribution of NF- κ B activity to protection from apoptosis, Nur77-expressing cells were pretreated with CAPE or KBK and subjected to different apoptotic stimuli. While Nur77-expressing cells showed high resistance to FasL, VP16 and TG treatment (Figure 4a), CAPE- and KBK-treated cells showed apoptosis levels similar to vector-only-transfected cells (Figures 4b and c). CAPE and KBK significantly reduced VP16 apoptosis in vector-only-transfected cell (Figures 4a–c), a phenomenon reported by other groups using different NF- κ B inhibitors.^{25,26} Since proteasome inhibition could affect pathways irrelevant to NF- κ B nuclear translocation, the I κ B α M super-repressor, which cannot be phosphorylated and degraded by the proteasome, thus sequestering NF- κ B in the cytosol, was used. Cells transiently transfected with I κ B α M showed levels of apoptosis similar to untreated cells or vector-only-transfected cells (Figures 4a, d and e), confirming that Nur77 antiapoptotic actions are independent of NF- κ B nuclear translocation. All inhibitors (CAPE, KBK and I κ B α M) significantly increased FasL + CHX apoptosis in vector-only stable transfectants, supporting the idea that short-lived antiapoptotic genes under NF- κ B control play a role in death receptor apoptosis. Since only DNA-binding NF- κ B inhibitors suppressed Nur77-mediated protection from cell death, this confirms that nuclear NF- κ B activity correlates with apoptosis inhibition.

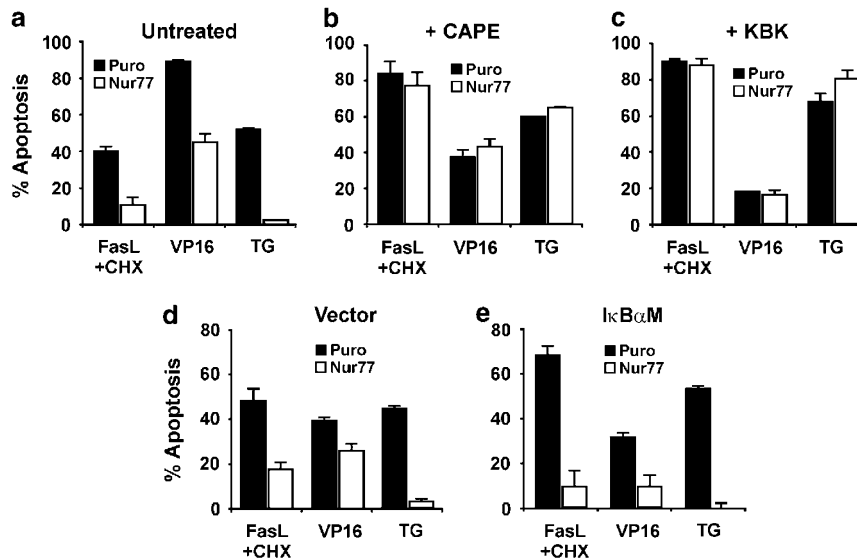


Figure 4 Nur77 inhibits apoptosis by nuclear NF- κ B activity modulation. HEK293 cells transfected with empty vector (puro) or Nur77 (Nur77) were preincubated for 24 h with (a) medium alone, (b) 100 μ M caffeic acid phenyl ester (CAPE) or (c) 2.5 μ g/ml kamebakaurin (KBK) and then treated for 48 h with 5 ng/ml FasL and 0.5 μ g/ml cycloheximide (CHX), 100 μ M etoposide (VP16) or 1 μ M thapsigargin (TG). Cells incubated with CAPE or KBK alone were used as controls for respective groups. The data represents mean \pm S.D. of three separate experiments. Cells were transiently transfected with (d) empty vector and pcDNA3.1-GFP5 or (e) the I κ B super-repressor I κ B α M and pcDNA3.1-GFP5 at a 10 : 1 DNA ratio and treated as in (a) for 48 h. Specific apoptosis of GFP-positive cells is expressed as the mean \pm S.D. of triplicates

Selective NF- κ B-driven gene induction by Nur77 overexpression

Nur77 expression clearly increased nuclear NF- κ B activity that was linked to protection from apoptosis. Since NF- κ B can contribute to the induction of both pro- and antiapoptotic factors, the expression of genes under NF- κ B control was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. This revealed that Nur77-expressing cells showed much higher constitutive expression of the NF- κ B-dependent antiapoptotic gene cIAP1, while Fas expression levels, which can also be modulated by NF- κ B,²⁷ did not vary significantly (Figure 5). KBK treatment, which inhibited Nur77-mediated increases in NF- κ B activity and protection from apoptosis, led to lower cIAP1 induction while having little effect on Fas expression (Figure 5). Thus, Nur77 appears to be able to selectively modulate gene expression under NF- κ B control.

Endogenous NGFI-B activity modulates apoptotic responses

The contribution of Nur77 to VP16 or TG apoptosis in other cell types has been shown by blocking endogenous Nur77 activity.^{28,29} Given results showing that Nur77 overexpression inhibits apoptosis with these agents, the contribution of endogenous NGFI-B family members was assessed. Deletion of Nur77 N-terminal domain required for transactivation and coactivator association generates a DN form of Nur77 that blocks the activity of all endogenous NGFI-B family members.¹⁴ Stable transfectants expressing dominant negative Nur77 (DN-Nur77) were treated with high doses of FasL (without CHX), VP16, CPT or TG. DN-Nur77 cells were highly sensitive to FasL-mediated death (Figure 6a), suggesting that inhibition of endogenous Nur77 activity sensitises cells to death receptor apoptosis, in agreement with results obtained with Nur77 overexpression. DN-Nur77 expression had no effect on cell death following exposure to DNA-damaging agents (Figure 6a), suggesting that low levels of endogenous Nur77 are insufficient to provide protection from CPT or VP16. These results are in line with data showing that the transfectants expressing several-fold higher Nur77 activity compared to endogenous Nur77 were not protected from

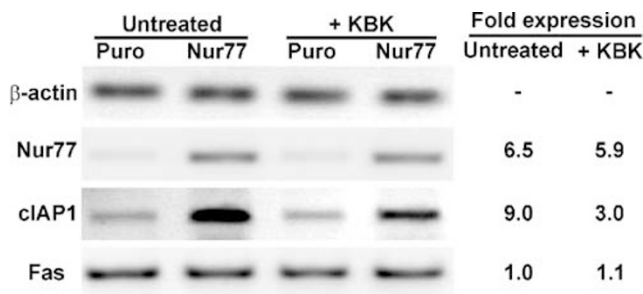


Figure 5 Nur77 promotes NF- κ B-dependent antiapoptotic gene induction. Stable cell lines were plated in six-well dishes (5×10^5 cells) and treated with 5 μ g/ml kamebakaurin (KBK) for 6 h. RT-PCR analysis was performed for cIAP1, Fas, Nur77 and β -actin. Integrated values of gel images were normalised against the β -actin signal and induction levels between vector only (puro) or Nur77 (Nur77) transfectants in untreated or KBK-treated cells were obtained by dividing normalised signal intensities

VP16-mediated apoptosis (Figure 2). Surprisingly, DN-Nur77 blocked TG-mediated apoptosis nearly as effectively as wild-type Nur77 (Figures 1a and 6a).

Given results implicating NF- κ B in Nur77-mediated protection from these agents, the impact of DN-Nur77 expression on NF- κ B activity was evaluated by gene reporter assays (Figure 6b). Cells transfected with DN-Nur77 showed both lower constitutive and VP16-induced NF- κ B activity, thus antiapoptotic gene transcription facilitated by endogenous Nur77 appears sufficient to provide protection from death receptor apoptosis, hence the increase in apoptosis observed upon FasL treatment. Inhibition of nuclear NF- κ B activity abrogated Nur77-mediated protection from ER stress apoptosis (Figures 4b and c). The fact that DN-Nur77 decreased NF- κ B activity but did not provide protection from TG-mediated cell death suggests that while NF- κ B activity can inhibit ER stress apoptosis, other events associated with NGFI-B family members are involved in the positive regulation of this death pathway.

Discussion

Nur77 is a mediator of apoptosis required for thymocyte negative selection^{12,30} and is involved in apoptosis in a variety of cellular contexts.^{17,28,31,32} Yet, Nur77 and related family

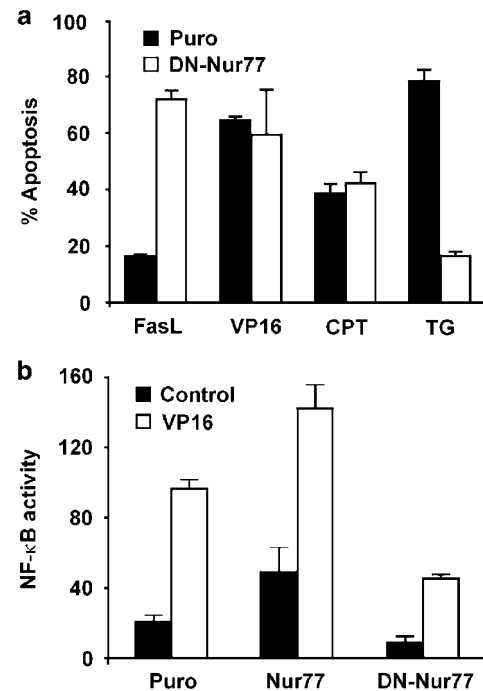


Figure 6 Contribution of endogenous Nur77 activity to cellular apoptosis. (a) 10^5 HEK293 cells transfected with empty vector (puro) or a dominant negative form of Nur77 (DN-Nur77) plated in 24-well dishes were treated with 50 ng/ml FasL, 100 μ M etoposide (VP16), 10 μ M camptothecin (CPT) or 1 μ M thapsigargin (TG) for 48 h. Data shown represent the mean specific apoptosis \pm S.D. of three different experiments. (b) Puro, Nur77 and DN-Nur77 transfectants were seeded in six-well dishes (5×10^5 cells per well) and transiently transfected with the NF- κ B reporter plasmid. At 24 h post-transfection, cells were treated with the DMSO carrier or 100 μ M etoposide (VP16) for 6 h. Data shown are the mean relative luminescence \pm S.D. of triplicates normalised on β -galactosidase activity

members were identified as growth factor inducible genes.^{2,5} Nur77 is expressed at high levels in cells derived from lung, breast and prostate tumours^{28,33,34} and its expression has been linked to increased proliferation of lung cancer cells.³⁵ Nur77 can also block cell death as it protects the A20 B cell line from ceramide-induced apoptosis and is a survival factor in TNF-induced apoptosis.^{19,20} Since Nur77 can participate in both pro- and antiapoptotic pathways, this work aimed at assessing the contribution of Nur77 to apoptosis when overexpressed or when endogenous NGFI-B family member activity was blocked.

While Nur77 overexpression in HEK293 cells had no noticeable effect on cell viability, it had profound effects on death induced by several apoptotic stimuli. Nur77 decreased apoptosis in response to DNA-damaging agents and low doses of FasL combined with CHX, and completely prevented ER stress apoptosis induced by TG. Nur77 overexpression was associated with enhanced NF- κ B activity and cIAP1 transcription. This enhancement was not caused by increased NF- κ B nuclear translocation since blocking I κ B degradation through proteasome inhibition with ALLN or by transfecting the I κ B super-repressor had no effect on Nur77-mediated increases in NF- κ B activity or apoptosis protection. NF- κ B activity could, however, be lowered by CAPE and KBK, inhibitors that form covalent adducts with NF- κ B family members and prevent DNA binding,^{23,24} arguing that the pool of nuclear NF- κ B is the target for enhancement associated with Nur77 overexpression. Inhibition of NF- κ B DNA binding rendered Nur77-expressing cells as sensitive as vector-transfected cells to etoposide or FasL combined with CHX and even more sensitive to TG. These results indicate that nuclear NF- κ B activity accounts for the antiapoptotic effects of Nur77.

Increased NF- κ B activity has been shown to protect cells from FasL, TNF and etoposide by indirectly suppressing caspase activity through cIAP1 and cIAP2 induction³⁶ and can protect from ER stress apoptosis.³⁷ Nur77 only provided protection from FasL-induced apoptosis at low ligand concentrations in the presence of CHX and protein synthesis inhibitors are known to reduce the levels of labile antiapoptotic factors, such as IAPs and cFLIP, which are under NF- κ B control.³⁸ Indirect NF- κ B-driven expression of short-lived antiapoptotic proteins by Nur77 should increase the threshold required for death signal transmission. Similar to what we observed with FasL, the TNF death pathway can be inhibited by Nur77 in the presence of CHX, but this was observed in cells with deficient NF- κ B pathways (TRAF2^{-/-} or RelA^{-/-}).²⁰ These results are in apparent opposition with what was observed with FasL, which requires NF- κ B activity for protection from apoptosis. However, other specific NF- κ B members such as c-Rel or RelB subunits might be modulated by Nur77 expression and the independence on proteosomal activity suggest that a non-classical pathway of activation is involved.

The mechanism by which Nur77 increases NF- κ B activity might be direct or indirect. It has been shown that Nur77 can interact directly with the NF- κ B p65/RelA subunit or indirectly through the SRC-1 transcriptional coactivator.³⁹ These interactions reduce Nur77 activity, suppress testicular steroidogenesis induced by TNF and favour NF- κ B recruitment to Nur77-containing promoters.³⁹ In this study, the NF- κ B-

binding site in the reporter used was derived from the ICAM-1 promoter.⁴⁰ It has been shown that Nur77 can inhibit IL-2 promoter activity by blocking a low-affinity NF- κ B site, which was promoter specific since substitution with an HIV LTR NF- κ B consensus site abrogated this inhibition.⁴¹ It is possible that Nur77 assists NF- κ B binding to promoters of antiapoptotic genes, while other NF- κ B responsive genes would be differently affected. Complex interactions between specific DNA elements, transcription factors and coregulators are likely to contribute to gene selectivity.

The antiapoptotic activity of Nur77 was evident under overexpression conditions, so high Nur77 expression by tumour cells would be expected to confer protection from various apoptotic stresses and increase cell survival. Indeed, elevated levels of Nur77 have been detected in radio-resistant B-CLL cells.⁴² In extraskeletal myxoid chondrosarcoma, the EWS/NOR1 oncogene arises by chromosomal translocation of the NOR-1 NGFI-B family member to EWS resulting in a highly expressed and potent transcriptional activator. Furthermore, high levels of NOR-1 and Nur1 have been detected in large cell lung carcinoma cells having enhanced metastatic potential.⁴³ Abnormally high expression of NGFI-B family members appears to provide a selective *in vivo* advantage. In addition, Nur77 and Nurr1 have been identified as potential oncogenes that can inhibit apoptosis in cancer cells.⁴⁴

In contrast, several reports have shown that loss of Nur77 expression or activity prevents apoptosis, suggesting that unlike the overexpressed form, endogenous Nur77 activity might be proapoptotic. To determine if basal Nur77 activity affects apoptosis of HEK293 cells, a DN construct able to block the function of all NGFI-B family members was used. Suppression of endogenous Nur77 activity strongly increased FasL-induced apoptosis, which was likely mediated by NF- κ B inhibition, since treatment with all chemical inhibitors or I κ B super-repressor expression caused a strong increase in FasL apoptosis in control cells. Furthermore, DN-Nur77 expression suppressed NF- κ B activity in untreated cells or cells treated with the NF- κ B inducer VP16. It appears that both endogenous and overexpressed Nur77 can protect from death receptor apoptosis, as suggested by others.²⁰

Conversely, the DN form of Nur77 decreased TG-induced cell death, indicating that endogenous Nur77 plays a role in ER stress apoptosis independently of its impact on NF- κ B activity. This is further supported by the fact that treatment of Nur77-expressing cells with KBK rendered them more susceptible to TG, suggesting that when NF- κ B activity is low or blocked, Nur77 may promote apoptosis. Nur77 was reported to be involved in apoptosis induced by TG²⁹ and calcium ionophore A23187, another ER stress inducer.¹⁷ While Nur77 might contribute to ER stress apoptosis, high expression levels did not cause spontaneous cell death of HEK293 cells. Other NGFI-B family members might contribute to the effects observed with DN-Nur77 in TG-mediated apoptosis since DN-Nur77 can functionally inactivate Nurr1 and NOR-1. Taken together, results indicate that NGFI-B family members such as Nur77 are multifunctional proteins that contribute to both positive and negative regulation of apoptosis. While Nur77 and related members are generally considered proapoptotic, our results support the idea that they should also be considered as important survival factors.

Materials and Methods

Plasmids

The human Nur77 cDNA cloned in pUC19 was obtained from Dr. J Drouin. Nur77 was amplified by PCR with primers 5' NurBamHI and M13 universal (Table 1). The DN Nur77 was generated by PCR truncation of amino acids 1–246 using primer Nur (246–598)¹⁴ (Table 1). Both forms of Nur77 were digested with BamHI and FspI, cloned into the BamHI/SmaI sites of pRSETc (Invitrogen Canada Inc, Burlington, ON, Canada), then subcloned in pRc/CMVpuro⁴⁵ along with the N-terminal poly-Histidine tag. GFP5 was generated by PCR mutagenesis to create the S65T-GFP5 variant described by Siemerling *et al.*⁴⁶ and cloned in pRSETc. Full-length Nur77 was first cloned in-frame in pRSET-GFP5, then the GFP5-Nur77 fusion or GFP5 were subcloned in pcDNA3.1(zeo) (Invitrogen). All sequences were verified on a CEQ 2000XL automated sequencer (Beckman Coulter, Mississauga, ON, Canada). pCMV-l κ B α M and pCMV β were from BD Biosciences (Nepean, ON, Canada). The NurRE-Luc reporter contains three repeats of the NurRE element⁴⁷ and pGL2-NF- κ B contains the NF- κ B-binding site from the human ICAM-1 promoter (segment –220 to +1) cloned in pGL2 Basic.⁴⁰

Reagents and cell lines

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Advanced DMEM (Invitrogen) supplemented with 5% heat-inactivated foetal calf serum (Sigma Chemical Co., St-Louis, MO, USA), 4 mM L-glutamine, and 100 U/ml penicillin–streptomycin (Invitrogen). Puromycin was from Sigma and VP16, CHX, TG, CPT, KBK and CAPE were from Calbiochem (La Jolla, CA, USA). Recombinant FasL (rhsSuperFasLigand™) was from Alexis Biochemicals (Carlsbad, CA, USA).

Transfections and gene reporter analysis

Transient transfections were performed with FuGene 6 (Roche Diagnostics, Laval, QC, Canada) on cells in logarithmic growth seeded in 24-well plates (10⁵ cells/well) or six-well plates (5 × 10⁵ cells/well) and cells were harvested 24–48 h later for analysis. For stable transfections, 1 μ g/ml puromycin was added 24 h post-transfection and clones were obtained by limiting dilution. Reporter assays were performed by transient transfection of NurRE-Luc or pGL2-NF- κ B luciferase reporters together with pCMV β at a 10 to 1 DNA ratio. Luciferase and β -galactosidase assays were performed according to the manufacturer's instructions (Promega Biosciences, Madison, WI, USA). Luminescence data were

collected on a Lumat LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN, USA) and normalised against β -galactosidase values.

Apoptosis analysis

Cells seeded in 24-well plates (10⁵ cells/well) were allowed to adhere for 24 h and treated for 24–48 h. Adherent cells were detached by 0.5% EDTA treatment, pooled with nonadherent cells, washed in PBS and analysed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Apoptosis was measured by cell size decrease with cells with low forward light scatter and normal side scatter being scored apoptotic.⁴⁸ For transient transfections, analysis was performed after gating on GFP-positive cells. Apoptosis was calculated using the formula: 100 × (% sample – % control)/(100 – % control).

RT-PCR analysis

Cells seeded in six-well plates (5 × 10⁵ cells/well) were allowed to adhere overnight before treatment. Total RNA was isolated with Trizol LS (Invitrogen) and first-strand synthesis was performed with SuperScript II (Invitrogen) according to the manufacturer's instructions. In total, 2 μ l of cDNA were used for PCR containing 1 × PCR buffer, 0.2 mM dNTP and 100 pmol of primers in a volume of 50 μ l. PCR reactions were performed in a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA) with primers specific to β -actin, Nur77, cIAP1 or Fas (Table 1). Cycling conditions were 30 s denaturation at 94°C, 30 s annealing at 55°C and 3 min elongation at 75°C for 25 cycles (β -actin and Nur77) or 30 cycles (cIAP1 and Fas). In all, 10 μ l of PCR reactions were electrophoresed on 2% agarose gels containing 0.1 μ g/ml ethidium bromide in Tris-Borate-EDTA. Gel images were captured with an AlphaImager 3400 system (Alpha Innotech, San Leandro, CA, USA) and signals were quantified using AlphaEase software (Alpha Innotech) and normalised against the β -actin signal.

Confocal microscopy

Cells seeded on glass coverslips in 24-well plates were transiently transfected and incubated 18–24 h prior to analysis. Mitochondria were stained 30 min with 100 nM Mitotracker Red (Invitrogen) prior to a 10 min fixation with 4% paraformaldehyde in PBS at 25°C. Coverslips were mounted with Geltol (Thermo Shandon Inc., Pittsburgh, PA, USA) plus 2.5% DABCO antifading reagent (Sigma), then slides were imaged on a Radiance 2000 confocal microscope (Bio-Rad Laboratories, Richmond, CA, USA).

Acknowledgements

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Table 1 Oligonucleotides used for PCR

Primer	Sequence 5' → 3'
5' NurBamHI	GGCAGGGGCAGCGGATCCCG
M13 universal	CGCCAGGGTTTTCCCAAGTCACGAC
Nur (246–598)	GCTTGGATCCCCCGTGACCTCAACCAAGGC
5' β -actin	GAGGGAAATCGTGCGTGACATCAA
3' β -actin	GGAACCGCTCGTTGCCAATAGTGA
5' Nur77	CGCACCTGTGAGGGCTGCAAG
3' Nur77	GGAGGCATCTGGGGGCTGCTTGGG
5' cIAP1	ATGAACATACTAGAAAACAGC
3' cIAP1	CCTGTCCTTTAATTCTTATCA
5' Fas	ATGCTGGGCATCTGGACCCTCCTA
3' Fas	TCTGCACTTGGTATTCTGGGTCCG

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Supplementary Information accompanies the paper on the Cell Death and Differentiation website (<http://www.nature.com/cdd>).