Neuron-specific Bcl-2 Homology 3 Domain-only Splice Variant of Bak Is Anti-apoptotic in Neurons, but Pro-apoptotic in Non-neuronal Cells*

Received for publication, November 16, 2000, and in revised form, January 26, 2001
Published, JBC Papers in Press, January 29, 2001, DOI 10.1074/jbc.M010419200

Yun-Fu Sun, Li-Ying Yu, Mart Saarma‡, Tönis Timmusk, and Urmas Arumäe§

From the Program of Molecular Neurobiology, Institute of Biotechnology, University of Helsinki, Viikki Biocenter, FIN-00014 Helsinki, Finland

We have identified and characterized N-Bak, a neuron-specific isoform of the pro-apoptotic Bcl-2 family member Bak. N-Bak is generated by neuron-specific splicing of a novel 20-base pair exon, which changes the previously described Bak, containing Bcl-2 homology (BH) domains BH1, BH2, and BH3, into a shorter BH3-only protein. As demonstrated by reverse transcription-polymerase chain reaction and RNase protection assay, N-Bak transcripts are expressed only in central and peripheral neurons, but not in other cells, whereas the previously described Bak is expressed ubiquitously, but not in neurons. Neonatal sympathetic neurons microinjected with N-Bak resisted apoptotic death caused by nerve growth factor (NGF) removal, whereas microinjected Bak accelerated NGF deprivation-induced death. Overexpressed Bak killed sympathetic neurons in the presence of NGF, whereas N-Bak did not. N-Bak was, however, still death-promoting when overexpressed in non-neuronal cells. Thus, N-Bak is an anti-apoptotic BH3-only protein, but only in the appropriate cellular environment. This is the first example of a neuron-specific Bcl-2 family member.

During development, two opposite processes, proliferation and naturally occurring cell death (apoptosis), regulate cell number in almost all tissues and organs (1). In the developing nervous system, for example, 30–80% of the initially produced neurons die, mostly due to deficiency of neurotrophic factors that neutralize the death program in neurons (2, 3). The cells of an organism retain a potential to die apoptotically during their entire lifetime (4). Apoptotic pathways are therefore delicately controlled by the proteins of the Bcl-2 family that are either anti-apoptotic or pro-apoptotic. When overexpressed, anti-apoptotic members protect cells against death stimuli, and their lack in vivo promotes developmental death or sensitivity to death stimuli. Conversely, pro-apoptotic Bcl-2 family members kill the cells even in the presence of life-promoting stimuli, and their deficiency reduces apoptotic death (7). All anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and Boo/DIVA) contain four conserved BH domains (BH1, BH2, BH3, and BH4), whereas pro-apoptotic members contain either three BH domains (BH1, BH2, and BH3) (Bax, Bak, and Mtd/Bok) or only the BH3 domain (Bad, Bid, Bik, Hrk, Bim, Rad9, and Noxa) (7–10). Interestingly, the recently discovered Bcl-G contains only BH3 and BH2 domains (11).

How the Bcl-2 family proteins work is still poorly understood. According to the current understanding, pro-apoptotic members containing several BH domains (Bax and Bak), when activated by a death stimulus, generate pores to the mitochondrial outer membrane that lead to the release of cytochrome c and other mitochondrial molecules to the cytoplasm, where they trigger activation of caspases at the apoptosome (5, 6). Once activated above certain threshold levels, caspases irreversibly execute cell death (6, 12). In addition, heterodimerization between certain pro- and anti-apoptotic Bcl-2 family proteins nullifies the activity of the partners, and the fate of the cell is determined by the member that is in excess (7). BH3-only proteins are believed to regulate or modulate the activity of multi-BH domain members. Thus, Bid activates Bax (13, 14), whereas Bad inactivates Bcl-xL (15), with the net result being cell death in both cases. Alternatively spliced transcripts have been described for many Bcl-2 family members, with the activity of the protein isoforms remaining unchanged in most cases (16). However, BH3-only splice variants encoding proteins with pro-apoptotic activity have been described for the anti-apoptotic Bcl-x (17) and Mcl-1 (18, 19) and also for Bcl-G (11). Here we describe a BH3-only splice variant of the pro-apoptotic Bak (20–24) and show that it is expressed exclusively in neurons and encodes a protein isoform that is anti-apoptotic in neurons, but promotes death in non-neuronal cells.

EXPERIMENTAL PROCEDURES
Cloning and Analysis of Bak Splice Variants—Full-length N-Bak and Bak cDNAs were generated by RT-PCR from P1 mouse brain RNA, inserted into the pCR3.1 expression vector (Invitrogen, Groningen, The Netherlands), and verified by sequencing. To check whether the expression plasmids produce the respective proteins in cells, COS-7 cells in semiconfluent 10-cm dishes were transfected with Bak- or N-Bak-encoding expression plasmids or with the empty pCR3.1 vector (5 μg/dish) using Fugene transfection reagent (Roche Molecular Biochemicals). To protect cells against death caused by overexpression of pro-apoptotic proteins, plasmid encoding human Bcl-xL (5 μg/dish) was cotransfected. Also, plasmid pGreenLantern-1, encoding green fluorescent protein (GFP) (0.5 μg/dish; Life Technologies, Inc.), was included to reveal

* This work was supported by Academy of Finland Programs 44896 (Finnish Center of Excellence Program 2000–2005) and 43679, European Union Biotech Grant BIO4–98-0293, and a Sigrid Juselius Foundation grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Biocentrum Helsinki Fellow.

§ To whom correspondence should be addressed: Program of Molecular Neurobiology, Inst. of Biotechnology, University of Helsinki, P. O. Box 56, Viikki Biocenter, FIN-00014 Helsinki, Finland. Tel.: 358-9-19159369; Fax: 358-9-19159366; E-mail: urmas.arumae@helsinki.fi.

1 The abbreviations used are: BH, Bcl-2 homology; P, postnatal day; E, embryonic day; RT-PCR, reverse transcription-polymerase chain reaction; GFP, green fluorescent protein; SCG, superior cervical ganglion/ganglia/ganglial; bp, base pair(s); RPA, RNase protection assay; NGF, nerve growth factor.
transfection efficiency. The next day, they were lysed in phosphate-buffered saline containing 10 mM KCl, 2 mM EDTA, 1% SDS, and protease inhibitors (Roche Molecular Biochemicals) and analyzed by Western blotting with anti-Bak antibodies (65606E, Pharmingen, San Diego, CA) (see Fig. 3A). The peptide sequence used to generate these antibodies is present in both Bak and N-Bak (25). Some transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.5% Triton X-100, and stained with the same anti-Bak antibodies. Both the Bak- and N-Bak-transfected cultures, but not the vector-transfected or untransfected cultures, contained strongly Bak-immunoreactive cells (data not shown). To demonstrate expression of both Bak protein, nuclei, mouse brain, or hippocampal neurons, or Neuro2a neuroblastoma cells were lysed in buffer containing either 2% Triton X-100 and 1% SDS or 1% SDS and 8 M urea. Either Bak proteins were immunoprecipitated from the lysates with the anti-Bak antibodies (Pharmingen 65606E), or the crude lysate was used, and the filter was probed with the same antibody. In all samples, only Bak (but not N-Bak) protein was visible (data not shown), whereas both N-Bak and Bak proteins were detected in control COS-7 cells transiently overexpressing N-Bak and Bel-1 (similar to the results shown in Fig. 3A). The same result was obtained with anti-Bak antibodies from Oncogene Research Products (AM04; Darmstadt, Germany) or from Santa Cruz Biotechnology (H-211; Santa Cruz, CA) (data not shown).

To show that the non-neuronal cells overexpressing N-Bak die apoptotically, HeLa cells were transiently transfected with expression vector for GFP-N-Bak or with the empty pEGFP-C1 vector. 2 h after transfection, GFP-N-Bak-transfected cells exhibited weak fluorescence, became round, and began to detach from the substrate, whereas GFP-expressing cells remained healthy and flat. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with 1 μM 4,6-diamidino-2-phenylindole. Images were acquired on an Olympus AX 70 Provis microscope and analyzed by Adobe Photoshop software.

For the DNA ladder assay, HeLa cells were transiently transfected with expression plasmids encoding N-Bak or GFP. After 3 h, DNA was isolated and analyzed for the presence of intranucleosomal degradation fragments according to a published protocol (26).

RT-PCR and RNase Protection—Total RNAs from different rat and mouse tissues or from cultured purified neurons or non-neuronal cells were isolated with Trizol reagent (Life Technologies, Inc.). RNAs from human tissues were obtained from CLONTECH. Rat cortical and hippocampal neurons were cultured as described (27). First strand cDNAs were synthesized using oligo(dT)15 or dN4 random primers (Roche Molecular Biochemicals) with enhanced avian reverse transcriptase (Sigma). cDNAs (1 μg) or Superscript II (Life Technologies, Inc.) were added to 1 μl cDNA and amplified by PCR with the High Fidelity PCR system (Roche Molecular Biochemicals) with enhanced avian reverse transcriptase (Sigma). PCR products (10–20 μl) were electrophoresed on a 1% agarose gel to reveal the 20-bp difference in the fragments. RT-PCR and RNase Protection assays were performed as described (29). Full-length N-Bak cDNA was used to generate 32P-labeled antisense RNA probe. The assay resulted in one fragment of 572 bp for N-Bak and two fragments of 322 and 230 bp for Bak. Only the 572- and 230-bp fragments are shown in Fig. 2. For a positive control, the mouse β-actin riboprobe (Ambion Inc., Austin, TX) was used.

**Neural Cultures**—P1–P2 mouse SCG were digested with collagenase (2.5 mg/ml; Worthington), dispase (5 mg/ml, Roche Molecular Biochemicals), and trypsin (10 mg/ml; Worthington) for 45 min at 37 °C and dissociated mechanically with a siliconized glass Pasteur pipette. Non-neuronal cells were removed by extensive preplating. Almost pure neurons were cultured in polynorhithine/laminin (Sigma)-coated 35-mm plastic dishes at a 1:1 ratio of nutrient mixture F-12 to Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 3% fetal calf serum (Hyclone, Cramlington, United Kingdom), serum supplement containing 35% bovine serum albumin (Pathotype, MN), 0.35% (v/v) Earle’s BSS (ICN Pharmaceuticals, Inc.), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite, and 340 ng/ml triiodothyronine (all from Sigma Chemical Co.) (30), and 30 ng/ml mouse 2.5 S nerve growth factor (NGF) (Promega, Madison, WI) at 37 °C in a humid atmosphere containing 5% CO2. Neither antibiotics nor antimicrobial drugs were included in the culture medium. Hippocampi from E16 mice were dissociated with trypsin (0.25%) for 15 min at 37 °C in Hanks’ balanced salt solution containing 1 mg/ml DNase I (Sigma) and 10 mM HEPES, pH 7.5, and dissociated with glass Pasteur pipettes, onto polyornithine (Sigma)-coated dishes, and grown further in neurobasal medium (Life Technologies, Inc.) containing B-27 serum substitute (Life Technologies, Inc.).

**Transfection of Cultured Primary Cells**—Nuclei of the SCG neurons, cultured for 5–6 days with 30 ng/ml NGF, were pressure-injected under direct visual control with expression plasmids encoding Bak or N-Bak or with the empty pCR3.1 vector, all 50 ng/μl. All injection solutions contained also 10 μg/μl pGreenLantern–1. A Model MNO-220 micromanipulator (Narishige International Ltd., London) and a Model S246 Transjector (Eppendorf Scientific, Westbury, NY) were used for injection. Neurons were grown further with NGF or in NGF-free medium with function-blocking anti-NGF antibodies (Roche Molecular Biochemicals). Initial neurons surviving the procedure were counted 3–4 h later. 50–100 neurons were successfully injected with each plasmid combination in every experiment. To later follow all injected neurons individually, the positions of the injected neurons were mapped according to the grid scratched in the bottom of the dish. Healthy fluorescent neurons with phase-bright cytoplasm and an intact neuritic tree, identified according to the fluorescence and the map, were counted daily and expressed as a percentage of the un.injected cells. Few neurons that lost fluorescence during experiment were subtracted from the initial neurons. Uninjected neurons were counted from one dish. Non-neuronal cells from dissociated P1 mouse SCG were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum without neurotrophic factors until all neurons were dead and injected as described above.

Neurons were cultured in polyornithine/laminin (Sigma)-coated 35-mm plastic dishes (all from Sigma Chemical Co.) (30), and 30 ng/ml mouse 2.5 S nerve growth factor (NGF) (Promega, Madison, WI) at 37 °C in a humid atmosphere containing 5% CO2. Neither antibiotics nor antimicrobial drugs were included in the culture medium. Hippocampi from E16 mice were dissociated with trypsin (0.25%) for 15 min at 37 °C in Hanks’ balanced salt solution containing 1 mg/ml DNase I (Sigma) and 10 mM HEPES, pH 7.5, and dissociated with glass Pasteur pipettes, onto polyornithine (Sigma)-coated dishes, and grown further in neurobasal medium (Life Technologies, Inc.) containing B-27 serum substitute (Life Technologies, Inc.).
RESULTS

N-Bak, a Novel Isoform of Bak, Is a BH3-only Protein.—When verifying the nucleotide sequences of RT-PCR fragments of Bak from mouse brain, we noticed a 20-bp sequence, GCCAGCAGCAACATGCACAG (Fig. 1A), not present in the published mouse Bak sequence (23). We cloned and sequenced full-length Bak cDNA from mouse brain. The 20-bp insert was found in several sequenced cDNA clones. Also some minor differences from the published Bak sequence were detected in all clones: there is an additional guanine between positions 147 and 148 as well as two additional cytidines between positions 153 and 154 of the published mouse Bak sequence. We do not know whether these minor differences result from sequencing errors of Ulrich et al. (23) or whether they represent polymorphisms or mutations in the Bak gene of the 3T3 cells used by Ulrich et al. to clone it. In the predicted Bak protein deduced from the corrected nucleotide sequence, residues 50 and 51 are both alanines, but not arginine and proline, as published by Ulrich et al. (23), followed by an additional alanine absent in the published sequence (Fig. 1D).

The 20-bp sequence is inserted at position 344 (corrected nucleotide numbering) of Bak cDNA (Fig. 1A), which corresponds exactly with the junction of exons 4 and 5 of the mouse Bak gene (23), suggesting that the 20-bp insert is a hitherto undescribed exon. We determined a partial sequence of the intron between exons 4 and 5 of the Bak gene. The 20-bp sequence was found in the intronic sequence flanked by intron-exon junction sequences corresponding to the GT-AG rule (Fig. 1C). In addition, the 3′-splice site of the 20-bp exon deviated from the respective consensus sequence (Pyr)12CAG (where Pyr is pyrimidine), with several purines interrupting the polypyrimidine tract (Fig. 1C). The majority of the alternatively spliced exons have weak 3′-splice sites with higher purine content compared with the 3′-sites of constitutively spliced exons (31, 32). We designated this exon as exon N (for neuron-specific exon; see below) and the transcript using this exon as N-Bak.

The corrected genomic organization of the mouse Bak gene and the two transcripts generated from it by alternative use of exon N are schematically presented in Fig. 1.

Use of exon N would cause a translational frameshift, resulting in a changed amino acid sequence and a truncated protein due to a premature stop codon (Fig. 1D). The predicted protein translated from N-Bak contains 150 amino acids, with a calculated molecular mass of 16.4 kDa and a calculated pI of 4.48. The previously described Bak isoform has 208 residues, with a calculated molecular mass of 23.3 kDa and a calculated pI of 6.08. The novel C-terminal amino acid stretch RPAATCTAYLRVASAGAAWLSWALTWPTSTSVV of N-Bak (Fig. 1D) has no homology to any of the known proteins. As predicted by the Sosui and TopPred2 programs, 24 C-terminal residues of this novel sequence (VASAGAAWLSWALTWPTSTSVS) may form a transmembrane α-helix. No other known structural motifs were found in this novel sequence by the PSORT II program. Use of exon N would lead to the change of the BH1 domain of the Bak protein into a different amino acid sequence. The BH2 as well as transmembrane domains would not be translated, whereas the BH3 domain would remain unchanged (Fig. 1D). Thus, use of exon N would convert the three BH domain-containing Bak protein into a BH3-only protein with a novel putative transmembrane domain. We designated the short protein isoform (encoded by N-Bak) as N-Bak and the previously known longer protein isoform as Bak.

A similar 20-bp sequence (GCCAGCAGCAACATGCACAG) was found in four independent Bak cDNA clones from human brain with two nucleotide differences from mouse exon N. This human sequence is inserted at the position identical to that in mouse Bak cDNA, leading to the same changes in amino acid sequence as in mouse N-Bak: a truncated protein of 150 amino acids, a novel C-terminal amino acid sequence (RPAATPTAYLRVASAGAAWLSWALTWPTSTSVV) with a predicted transmembrane domain, and a lack of BH1 and BH2 domains.

N-Bak Transcripts Are Expressed in Neurons, but Are Absent in Non-neuronal Cells—Expression of Bak splice variants in different tissues was studied by RT-PCR and RPA. RT-PCR analysis showed that Bak was expressed in all studied rat tissues, whereas N-Bak was expressed exclusively in the nervous tissue (Fig. 2A). Identical results were obtained when RNAs from human tissues were analyzed by RT-PCR (data not shown). RPA also revealed expression of Bak transcripts in all adult mouse tissues analyzed, whereas N-Bak was detected only in brain. The levels of both Bak and N-Bak transcripts were rather similar in different brain regions (Fig. 2B). Furthermore, Bak and N-Bak transcripts were differently regulated during mouse brain development. Low levels of N-Bak
Neuron-specific Anti-apoptotic Bak Isoform

Fig. 2. Expression of N-Bak and Bak transcripts in rat and mouse tissues and cells. A, RT-PCR analysis of Bak transcripts in rat tissues and cultured cells. B, RPA analysis of Bak transcripts in non-neuronal tissues and brain regions of adult mouse. Shown are 572-bp fragments of N-Bak and 230-bp fragments of Bak. C, RPA analysis of Bak transcripts in mouse brain of different ages. Shown are 572-bp fragments of N-Bak and 230-bp fragments of Bak. The lower panels in B and C show the levels of β-actin transcripts in the RNA samples analyzed. Note that β-actin mRNA levels decreased during postnatal brain development, as has been reported (71). D, RT-PCR analysis of Bak and N-Bak expression in cultured neonatal mouse SCG and hippocampal neurons free of non-neuronal cells, non-neuronal SCG and hippocampal cells free of neurons, or undissociated hippocampal tissue (Total). Note that the RT-PCR analyses were not quantitative and show only the presence or absence of the transcripts analyzed. ctx, cortex; ctx g, cortical non-neuronal cells; hip, hippocampus; sp c, spinal cord; sc n, sciatic nerve of adult rat; ad, adult; hrt, heart; kid, kidney; lie, liver; mus, skeletal muscle; spl, spleen; tes, testis; thy, thyroid; thym, thymus; olf, olfactory bulb; str, striatum; tha, thalamus; col, colliculi superior and inferior; v midbr, ventral midbrain; cbl, cerebellum; med, medulla; total, whole P60 brain; rRNA, yeast tRNA.

were present at E13, and the levels increased in the late embryogenesis and early postnatal days, with the peak being around birth (Fig. 2C). In contrast to this, the levels of Bak were high in E13 brain and decreased gradually during development (Fig. 2C).

Both transcripts of Bak were detected in cultured cells of rat cerebral cortex and hippocampus (Fig. 2A) that contained neurons as well as non-neuronal cells. In contrast, the non-neuronal cells from rat cerebral cortical culture expressed only Bak, but not N-Bak. Similarly, the rat sciatic nerve, known to contain mostly Schwann cells, expressed Bak, but not N-Bak (Fig. 2A). To clarify the cell-type specificity of expression of the two Bak transcripts, we manually separated cultured neonatal mouse SCG or E16 mouse hippocampal neurons from all non-neuronal cells (see “Experimental Procedures”). Neuron-free cultures of non-neuronal cells from both sources were also prepared. We then analyzed expression of Bak splice variants in purified cell populations by RT-PCR. Both SCG and hippocampal neurons expressed only N-Bak, but not Bak. Conversely, only Bak (but not N-Bak) was expressed in non-neuronal cells (Fig. 2D). The experiments with purified SCG neurons were repeated three times with identical results. Absence of Bak in neurons was further verified with totally non-neuronal cell-free SCG neurons by nested PCR. Bak message was still not detected (data not shown). Although we did not analyze other neuronal populations, it is probable that the Bak transcript in brain tissues (Fig. 2C) may be of glial origin. Thus, expression of N-Bak is strictly neural tissue-specific and, at least in SCG and hippocampus, strictly neuron-specific, whereas Bak is expressed almost ubiquitously (20, 23), but is absent (or below the detection limit) in the neurons. Exon N, as well as neuron-specific expression of N-Bak, is conserved in the mouse, rat, and human species. However, as our attempts to demonstrate endogenous N-Bak protein failed (see “Experimental Procedures”), we do not have evidence that endogenous N-Bak mRNA is translated into protein in neurons.

N-Bak Is a Survival-promoting Protein in Primary Neurons, but a Death-promoting Protein in Non-neuronal Cells—To study the functional activity of N-Bak in its natural cellular environment, we microinjected cultured neonatal mouse sympathetic SCG neurons with the expression plasmid encoding N-Bak or Bak and maintained the neurons further with or without NGF. Cultured neonatal SCG neurons are known to die apoptotically when deprived of NGF (33). The respective proteins were produced from the corresponding expression plasmids in transiently transfected COS-7 cells as shown by Western blotting (Fig. 3A). An ~28-kDa band was greatly enhanced in Bak-transfected cells, comigrating with the endogenous Bak in COS-7 cells, whereas a smaller band of ~22 kDa was revealed only in N-Bak-transfected cells (Fig. 3A). For some unknown reason, both proteins migrated somewhat slower than predicted by their amino acid sequences. N-Bak was also produced by the injected expression plasmid in the neurons, as shown by staining the injected neurons with anti-Bak antibodies (Fig. 3B). As shown previously (34), overexpressed Bak rapidly killed the neurons even in the presence of NGF (Fig. 4A). In contrast, 93.7 ± 2.4% (mean ± S.E.) of the NGF-maintained N-Bak-expressing neurons remained healthy, which does not differ significantly from vector-injected or uninjected neurons (Fig. 4A). The same percentage of neurons survived when the concentration of injected N-Bak-encoding plasmid was raised from 50 to 100 ng/μl (data not shown). When deprived of NGF, 19.4 ± 6.7% of the uninjected neurons and 17.8 ± 8.6% of the vector-injected neurons remained alive 72–75 h after injection (Fig. 4B). By that time, overexpressed Bak had killed almost all neurons (Fig. 4B), as reported previously (21). In contrast, 78.0 ± 2.2% of the N-Bak-expressing neurons resisted NGF removal, which is statistically significantly different from the vector-injected (p < 0.001) and uninjected (p < 0.001) neurons (Fig. 4B). A similar percentage of neurons were maintained alive when the concentration of the N-Bak-encoding plasmid was raised to 100 ng/μl (data not shown). Thus, in neurons, alternative splicing converts pro-apoptotic Bak into anti-apoptotic N-Bak. To our knowledge, this is the first demonstration of the anti-apoptotic activity of a BH3-only protein.

The survival-promoting activity of N-Bak was unexpected since we had noted earlier that its overexpression triggers death of transfected COS-7 cells (see “Experimental Procedures”). To address this controversy, we studied the effects of overexpressed Bak isoforms in non-neuronal cells more closely. To this end, we microinjected expression plasmids encoding...
Bak, N-Bak, or empty vector into primary non-neuronal cells of dissociated P1 mouse SCG. These cultures, containing mostly glial cells, but also epithelial and fibroblastic cells, expressed mRNA for \textit{Bak}, but not for N-\textit{Bak} (Fig. 2D). 24 h after injection, 90.7 ± 1.2% (mean ± S.E.) of the vector-injected control cells had survived, showing that these cells tolerate well the procedure and ectopic DNA. As expected, only 10.6 ± 1.3% of the

\textbf{FIG. 3.} Transfection of cells with expression plasmid encoding N-Bak. A, COS-7 cells were transfected with plasmids encoding Bak, N-Bak, or empty vector together with Bcl-xL and GFP-encoding plasmids. Cell lysates were analyzed by Western blotting with anti-Bak antibodies. Molecular mass markers are shown on the left. The anti-Bak antibodies recognize the introduced Bak (~28 kDa) \textit{(first lane)}, which comigrated with the endogenous Bak of the COS-7 cells, and the introduced N-Bak (~22 kDa) \textit{(second lane)}. Note that direct comparison of the intensities of Bak bands between Bak-transfected and mock-transfected or untransfected cells is inappropriate, as Bak and N-Bak killed many cells despite the presence of Bcl-xL. B, shown is the expression of the introduced N-Bak protein in microinjected SCG neurons. Cultured neonatal mouse SCG neurons were injected with a mixture of expression plasmids encoding N-Bak and GFP or empty vector and GFP, grown 3 days with NGF, and stained with anti-Bak antibodies. Bak immunoreactivity was visualized by rhodamine-conjugated secondary antibodies. Three images \textit{(phase-contrast, green fluorescence for GFP, and red fluorescence for Bak immunoreactivity)} were captured for every neuron. Shown are one typical Bak/GFP-injected neuron, one typical vector/GFP-injected neuron, and one typical uninjected neuron. Faint red fluorescence of control cells is a nonspecific background.

\textbf{FIG. 4.} Effect on survival of overexpression of \textit{Bak} splice variants in primary SCG cells. A and B, neonatal mouse sympathetic neurons were microinjected with expression plasmids encoding Bak, N-Bak, or the empty pCR3.1 vector \textit{(all 50 ng/µl)} together with pGreen-Lantern-1 \textit{(10 ng/µl)} and grown further with \textit{(A)} or without \textit{(B)} NGF. Living fluorescent neurons, counted 72–75 h after injection, are expressed as percentage of initial cells counted 3–5 h after injection. C, non-neuronal cells from neonatal mouse SCG were injected as described for A and B, and living fluorescent cells were counted 24 h after injection. Means ± S.E. of three independent experiments are shown for each condition. Statistical significance between means was estimated by one-way analysis of variance. *, p < 0.05; ***, p < 0.001.
**Neuron-specific Anti-apoptotic Bak Isoform**

We describe here N-Bak, a splice variant of Bak, that is generated by the use of a novel exon. Insertion of this exon causes a translational frameshift in Bak mRNA, thereby changing the C-terminal amino acid sequence of the protein product. In N-Bak, only some structural elements of Bak, including the BH3 domain, are preserved, whereas the BH1, BH2, and BH4 domains, as well as the predicted pore-forming hydrophobic a6- and a6-helices, are missing due to a translational frameshift and premature stop codon. Splice variants encoding different protein isoforms have been described for several other Bcl-2 family members. Indeed, a similar distribution pattern was observed for GFP-N-Bak (Fig. 5), suggesting that GFP-N-Bak is localized to intracellular membranes. Indeed, a similar distribution pattern was observed for GFP-Bcl-2 (Fig. 5), which is known to be associated with intracellular membranes (35). Deletion of the putative transmembrane domain abolished the clustered localization of GFP-N-Bak. Instead, a diffuse cytosolic pattern was observed that was similar to the localization of GFP (Fig. 5). Thus, the predicted transmembrane domain of N-Bak is used in SCG neurons to anchor N-Bak to intracellular membranes. Green fluorescent clusters overlapped with Mitotracker Red CMXRos in GFP-N-Bak- and GFP-Bcl-2-expressing neurons. However, Mitotracker was not specifically concentrated in the clusters of GFP-N-Bak and GFP-Bcl-2 (data not shown). More studies are necessary to ascertain the identity of neuronal membranes associated with clustered localization of GFP-N-Bak.

**DISCUSSION**

We describe here N-Bak, a splice variant of Bak, that is generated by the use of a novel exon. Insertion of this exon causes a translational frameshift in Bak mRNA, thereby changing the C-terminal amino acid sequence of the protein product. In N-Bak, only some structural elements of Bak, including the BH3 domain, are preserved, whereas the BH1, BH2, and BH4 domains, as well as the predicted pore-forming hydrophobic a6- and a6-helices, are missing due to a translational frameshift and premature stop codon. Splice variants encoding different protein isoforms have been described for several other Bcl-2 family members. In most cases, as for Bcl-xS (37), Bcl-xL (37), Bcl-xATM (38), Bax-α (39), Bax-α (40), Bax-α (41), Bcl-2β (42), and Bad-β (43), the changes involve the C-terminal regions of the proteins, leaving the BH domains unaltered. In other splice variants, regions encoding one or more BH domains are missing. For example, Bax-ε (44) lacks the BH2 domain; Bax-δ lacks the BH3 domain (45); and, BokS contains only the BH2 domain and a composite domain consisting of half of the BH3 domain and half of the BH1 domain (46). The functional activity of these splice variants remains mainly unchanged. The pro-apoptotic activity of three splice variants of Bim is increased as the protein is shortened (47). There are, however, a few cases where alternative splicing generates shorter BH3-only variants of multi-BH domain Bcl-2 family proteins, whose activity is converted opposite to that of the longer isoform. So far, such splice variants have been described only for the anti-apoptotic proteins Bcl-xL (17) and Mcl-1 (18, 19). Thus, the multi-BH domain Bcl-xL and Mcl-1L isoforms protect against apoptosis, whereas their short BH3-only variants, Bcl-xS, which, however, possesses also a BH4 domain and Mcl-1S, promote death. The recently described Bcl-G has also two splice variants: Bcl-GF, which contains the BH3 and BH2 domains and which has only minor effect on apoptosis, and Bcl-Gs, a BH3-only protein with strong death-promoting activity (11). In contrast, N-Bak, a BH3-only variant of the pro-apoptotic protein Bak described here, is anti-apoptotic in cell types in which it is endogenously expressed. Thus, the change of activity to the opposite direction may be a general phenomenon when a multi-BH domain Bcl-2 family protein is converted into a BH3-only protein by alternative splicing.

The change in function of Bcl-xS and Mcl-1L isoforms protect against apoptosis, whereas their short BH3-only variants, Bcl-xS, which, however, possesses also a BH4 domain and Mcl-1S, promote death. The recently described Bcl-G has also two splice variants: Bcl-GF, which contains the BH3 and BH2 domains and which has only minor effect on apoptosis, and Bcl-Gs, a BH3-only protein with strong death-promoting activity (11). In contrast, N-Bak, a BH3-only variant of the pro-apoptotic protein Bak described here, is anti-apoptotic in cell types in which it is endogenously expressed. Thus, the change of activity to the opposite direction may be a general phenomenon when a multi-BH domain Bcl-2 family protein is converted into a BH3-only protein by alternative splicing.

The change in function of Bcl-xS and Mcl-1L isoforms protect against apoptosis, whereas their short BH3-only variants, Bcl-xS, which, however, possesses also a BH4 domain and Mcl-1S, promote death. The recently described Bcl-G has also two splice variants: Bcl-GF, which contains the BH3 and BH2 domains and which has only minor effect on apoptosis, and Bcl-Gs, a BH3-only protein with strong death-promoting activity (11). In contrast, N-Bak, a BH3-only variant of the pro-apoptotic protein Bak described here, is anti-apoptotic in cell types in which it is endogenously expressed. Thus, the change of activity to the opposite direction may be a general phenomenon when a multi-BH domain Bcl-2 family protein is converted into a BH3-only protein by alternative splicing.

The change in function of Bcl-xS and Mcl-1L isoforms protect against apoptosis, whereas their short BH3-only variants, Bcl-xS, which, however, possesses also a BH4 domain and Mcl-1S, promote death. The recently described Bcl-G has also two splice variants: Bcl-GF, which contains the BH3 and BH2 domains and which has only minor effect on apoptosis, and Bcl-Gs, a BH3-only protein with strong death-promoting activity (11). In contrast, N-Bak, a BH3-only variant of the pro-apoptotic protein Bak described here, is anti-apoptotic in cell types in which it is endogenously expressed. Thus, the change of activity to the opposite direction may be a general phenomenon when a multi-BH domain Bcl-2 family protein is converted into a BH3-only protein by alternative splicing.

The change in function of Bcl-xS and Mcl-1L isoforms protect against apoptosis, whereas their short BH3-only variants, Bcl-xS, which, however, possesses also a BH4 domain and Mcl-1S, promote death. The recently described Bcl-G has also two splice variants: Bcl-GF, which contains the BH3 and BH2 domains and which has only minor effect on apoptosis, and Bcl-Gs, a BH3-only protein with strong death-promoting activity (11). In contrast, N-Bak, a BH3-only variant of the pro-apoptotic protein Bak described here, is anti-apoptotic in cell types in which it is endogenously expressed. Thus, the change of activity to the opposite direction may be a general phenomenon when a multi-BH domain Bcl-2 family protein is converted into a BH3-only protein by alternative splicing.
Neuron-specific Anti-apoptotic Bak Isoform

explanation, as their domain arrangement is similar to that of BH3-only proteins. Based on the determined three-dimensional structures of Bid (36, 48) and Bcl-xL, with or without a bound BH3 domain of Bak (49–51), a ligand-receptor model has been proposed for the heterodimerization of pro- and anti-apoptotic Bcl-2 family proteins (8, 36). The BH3-only proteins (and also activated Bax and Bak) expose their BH3 domains at the surface (so-called ligand state), whereas in the anti-apoptotic family members, the BH1, BH2, and BH3 domains form a hydrophobic surface groove (a receptor state). Pro-apoptotic members insert their BH3 domains into the surface pocket of the anti-apoptotic partners, thereby inactivating them and favoring death. In Bcl-xL and Mcl-1S, hydrophobic grooves cannot form due to loss of the BH1 and BH2 domains. Instead, Bcl-xS is predicted to take a ligand conformation with an exposed BH3 domain (36). Although we do not know the spatial structure of N-Bak, it probably has a conformation similar to that of Bcl-xS and should be intrinsically pro-apoptotic. Indeed, N-Bak was strongly pro-apoptotic in non-neuronal cells. This is, however, an artificial situation, as N-Bak is not endogenously expressed in these cells.

In neurons, in which N-Bak is endogenously expressed, it has no intrinsic apoptosis-promoting activity. Apparently, something in the neuronal environment blocks the killing activity of N-Bak. In neurons that would otherwise die due to the absence of NGF signaling, overexpressed N-Bak actively neutralized the death program. A survival-promoting effect of N-Bak in neurons was surprising, as to our knowledge, there are no other examples where a BH3-only protein protects the cells against apoptosis. Actually, the BH3 domain has intrinsic apoptosis-promoting properties, being called a minimal death domain. Indeed, oligopeptides encompassing little more than the BH3 domain of Bak are sufficient to release cytochrome c from isolated mitochondria, to activate caspases, and to promote cell death (52–55). The same was shown for the BH3 domains of Bax, Bid, Bad, and Bik (15, 52, 54, 56, 57). Moreover, the BH3 domains of the anti-apoptotic Bcl-2 and Bcl-xL also facilitate apoptosis (58). How then can overexpressed N-Bak with an intact BH3 domain protect neurons from apoptotic death? Binding to and neutralizing the long Bak isoform, as shown for Bcl-xL/Bcl-xS and Mcl-1S/Mcl-1S (18, 19, 58), are not probable in SCG neurons, as they do not express Bak (although very low levels of Bak transcripts may still remain undetected by our assay). Heterodimerization with other Bcl-2 family members, e.g. neutralizing Bax or releasing Bcl-xL, from some blocking constraint, cannot be excluded. However, we have not found any difference in the expression of other Bcl-2 family members in neurons and non-neuronal cells of cultured mouse SCG. Instead, almost all the known Bcl-2 family members were found in both cell types. Therefore, we do not believe that simple heterodimerization with known Bcl-2 family members can explain the opposite behavior of N-Bak in neurons and non-neuronal cells. We propose that N-Bak is intrinsically apoptosis-promoting, as predicted by its domain structure, but that in neurons, it protects against apoptosis indirectly via some other neuron-specific molecules. The molecular interactions of N-Bak in neurons are currently being studied in our laboratory.

We are aware that the study of a protein overexpressed at high levels does not necessarily reflect the functional properties of the endogenous protein. Moreover, our failure to demonstrate endogenous N-Bak protein suggests that the levels of the protein may be very low, in contrast to highly overexpressed N-Bak in our microinjection experiments. Therefore, we cannot claim that endogenous N-Bak is also anti-apoptotic in neurons.

N-Bak is expressed strictly in the nervous tissue and, at least in SCG and hippocampus, strictly in neurons and not in non-neuronal cells. This is, to our knowledge, the first neuron-specific member of the Bcl-2 family. Our results call for caution when interpreting functional studies of Bcl-2 family proteins in an inappropriate cellular environment. Indeed, overexpressed N-Bak had an opposite effect in different cell types. Several other Bcl-2 family members regulate life and death preferentially in some (but not all) tissues. Bid is the main mediator of Fas-induced death in hepatocytes, but not in thymocytes (66), whereas the primary cells affected by the absence of Bim are T and B lymphocytes (67). Bax-γ, a pro-apoptotic splice variant of Bax, is expressed mostly in immune organs and plays a critical role in T cell survival after T cell receptor activation (37). Expression of Bcl-xL is more restricted than that of Bcl-xS (68). Overexpressed Bcl-xL promotes apoptosis in 3T3 cells (69), whereas in 293 kidney epithelial cells or yeast cells, it only neutralizes the survival-promoting effect of Bcl-XL but is not apoptotic by itself (58). Interestingly, whereas Bcl-Gx is expressed in many tissues, its BH3-only isoform is exclusively testis-specific (11).

The function of N-Bak in the nervous system remains elusive, but a specific role is anticipated by its strict neuron-specific expression. Characterization of the nervous system of Bak-deficient mice has not yet been reported (70). Transcripts of N-Bak are expressed at the highest levels in perinatal and newborn mouse brain, at the time of active neurogenesis and programmed cell death. Presumably, N-Bak is part of the molecular network controlling the delicate processes that regulate neuronal number in the developing nervous system, but its explicit role remains to be studied.

Acknowledgments—We thank Dr. Alexei Titievsky and Mikhail Pavliev for assistance with confocal microscopy, Dr. Deping Guo for preparing the GFP-N-Bak-ΔTM construct, Dr. Susanne Hamnér (Biomedical Center, Uppsala) for providing the GFP-Bcl-2 construct, and Dr. Matti Airaksinen for critical reading of the manuscript.

REFERENCES

1. Jacobsen, M. D., Weil, M., and Raff, M. C. (1997) Cell 88, 347–354
2. Oppenheimer, R. W. (1991) Annu. Rev. Neurosci. 14, 543–561
3. Yuan, J., and Korsmeyer, S. J. (1999) Nature 396, 18–21
4. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell 96, 245–254
5. Green, D. R. (2000) Cell 102, 1–4
6. Hengartner, M. O. (2000) Nature 407, 770–776
7. Antonsen, B., and Martinou, J.-C. (2000) Exp. Cell Res. 256, 50–57
8. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) Genes Dev. 13, 1899–1911
9. Komatsu, K., Miyaishi, T., Hang, H., Hopkins, K. M., Zheng, W., Cuddeback, S., Yamada, M., Lieberman, H. B., and Wang, H.-G. (2000) Nat. Cell Biol. 2, 1–6
10. Oda, E., Okhi, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) Science 288, 1053–1058
11. Guo, B., Godzik, A., and Reed, J. C. (2001) J. Biol. Chem. 276, 2780–2785
12. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052

Y.-P. Sun, unpublished observation.
Neuron-specific Bcl-2 Homology 3 Domain-only Splice Variant of Bak Is Anti-apoptotic in Neurons, but Pro-apoptotic in Non-neuronal Cells
Yun-Fu Sun, Li-Ying Yu, Mart Saarma, Tõnis Timmusk and Urmas Arumäe

J. Biol. Chem. 2001, 276:16240-16247.
doi: 10.1074/jbc.M010419200 originally published online January 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010419200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 70 references, 31 of which can be accessed free at
http://www.jbc.org/content/276/19/16240.full.html#ref-list-1