

Research Article

Membrane translocation and oligomerization of hBok are triggered in response to apoptotic stimuli and Bnip3

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Abstract. hBok is a human pro-apoptotic member of the Bcl-2 family. By fluorescence in situ hybridization and in silico analysis, hBok was found to be located on chromosome 2q37.3. Its expression was detected in various organs and several hormonally regulated cancer cells. Expression of hBok was shown to be upregulated in estrogen-dependent breast cancer by estrogen deprivation and in myocardial cells during hypoxia. Confocal laser scanning microscopy examinations and subcellular fractionation studies showed that hBok was distributed in both the

cytosol and intracellular membranes of healthy cells. Upon overexpression of hBok or stimulation of apoptosis, hBok became integrated into the membrane. Furthermore, apoptosis and oligomerization were promoted by BH3-only proteins, such as Bid, Bnip3 and p53, but prevented by BFL-1. hBok was found to interact with Bnip3. Our findings suggest that functional BH3-only proteins facilitate the oligomerization and insertion of hBok into the membrane to activate it.

Key words. hBok; apoptosis; Bnip3; subcellular localization; oligomerization.

Introduction

The Bcl-2 family proteins are crucial regulators of apoptosis, in which they exert direct control through the release of apoptogenic factors, such as cytochrome c, to activate the proteolytic caspase cascade [1]. In mammalian cells, at least 20 different Bcl-2-related proteins have already been identified [2, 3]. Based on functional studies and the retention of BH ('Bcl-2 homology') domains, the Bcl-2 family is divided into three subgroups: (i) the Bcl-2 subgroup, containing more than one BH domain, including all anti-apoptotic proteins, such as Bcl-2, Bcl-xL, A1/Bfl-1

and Mcl-1; (ii) the Bax subgroup consisting of multi-BH domain pro-apoptosis members, such as Bax, Bak, Bok and Bcl-rambo [4–6]; (iii) the third subgroup containing BH3-only proteins, such as Bid and Bim, which can interact with either anti-apoptotic proteins or pro-apoptosis members and promote apoptosis [4].

BH3-only proteins are important in facilitating oligomerization of Bax and Bak leading to pore formation in the mitochondrial membrane allowing the release of cytochrome c [7–9]. Bax has also been reported to kill cells by a caspase-independent mechanism [10]. Although knockout studies in mice revealed that inactivation of either *bax* or *bak* alone has little consequence, while elimination of both genes dramatically impairs developmental apoptosis in many tissues [11], recent studies have shown that in some tissues, the activation of Bak requires

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Bax [12]. These results suggest that the regulation of Bax and Bak interaction may not be simply redundant [13]. The existence of other members of the Bax subfamily further implies that modulation of apoptosis may be controlled by more than one mechanism [14]. Indeed, recent studies revealed that oligomerization of both Bax and Bak could be stimulated by p53, but the latter can only interact with Bak [15] and not with Bax [16]. Therefore, p53 functions also as a BH3-only-like protein.

The mechanisms of action of other multi-domain proteins, such as Bok, and their contribution to the developmental susceptibility of apoptosis have not yet been established. The human Bok gene (*hBok*) has been identified together with its rat and mouse counterparts, *rBok* and *Mtd* [4, 5, 17]. Rat Bok was shown to be expressed exclusively in reproductive tissues [4]. In this study, we show that hBok originally identified in human granulosa cells is expressed more widely in other tissues. We have further characterized the expression and regulation of human Bok. We also investigated in detail both the activation of human Bok and its mode of action.

Materials and methods

Chromosomal localization of hBok by fluorescence in situ hybridization and partial sequencing

The molecular cloning of hBok was described previously with the GenBank accession number AF174487 [5]. A P1 DNA was isolated by PCR screening of P1 Human Libraries (Genome Systems, St. Louis, M.) with

primers 5'-CCTCGCGGGTCTGAATGGAAGG-3' and 5'-CGAGCGGTCAAAGGCGTCCAT-3' according to the manufacturer's recommended protocol and sequenced partially. Fluorescence in situ hybridization (FISH) was performed on human metaphase chromosomes. The *bok* PAC DNA probe was labeled with digoxigenin-11-dUTP using a nick translation kit (Roche, Basel, Switzerland), and ethanol-precipitated with salmon sperm DNA and *Escherichia coli* tRNA as described previously [18]. Hybridization and detection of fluorescence signals were performed accordingly. Fluorescence images were observed by an Olympus BX-60 epifluorescence microscope with Olympus filter (Olympus, Tokyo, Japan) set U-MWIB (excitation at 460–490 nm), U-MSWG (480–550 nm) and U-MWU (330–385 nm). The gene was located to human chromosome 2q37.2 using a query of the sequence to the NCBI STS database and the Human, Mouse Genome Browser program. PCP was used to determine the sizes of introns.

Plasmid constructions

The original PCR products amplified from human granulosa cDNA were cloned in the *EcoRI/NotI* site of vector pZero-1 (Invitrogen, Groningen, The Netherlands) and sequenced [5]. An EST clone (IMAGE 32090) was also obtained from the collection of the IMAGE Consortium. Full-length hBok cDNA was further subcloned into the pLEGFP-c1 vector (Clontech, Palo Alto, LA, USA) in the *HindIII/BamHI* site. Forward and reverse primers are listed in table 1. The constructs were confirmed by sequencing.

Table 1. PCR primers used to generate fragments for hBok constructs and to detect mouse Bcl2-related gene expression

			PCR product size (bp)
GFP-hBok	F	5'-TCTCGAGCAAGCTTGAATTCTATGGAGGTGCTGC GGCGTCCTCGGT-3'	636
	R	5'-CGGGATCCTCATCTCTCTGGCAGCAGCACGAAG-3'	
hBok	F	5'-CCTCGCGGGTCTGAATGGAAGG-3'	164
	R	5'-CGAGCGGTCAAAGGCGTCCAT-3'	
mBok	F	5'-CTCCCTCACTCAAATTGGGA-3'	267
	R	5'-ACTGAGGCCAGCTCATGTCT-3'	
mNip3	F	5'-GGGTTTTCCCAAAGGAATA-3'	179
	R	5'-GACCACCAAGGTAATGGTG-3'	
mBad	F	5'-TTCCAGATCCCAGAGTTTG-3'	488
	R	5'-GGAGATCACTGGGAGGGGTGG-3'	
mNoxa	F	5'-CGTCGGAACGCGCCAGTGAACCC-3'	335
	R	5'-TCCTTCCTGGGAGGTCCCTTCTTGC-3'	
mBmf	F	5'-CCCTTGGGGAGCAGCCCCCTG-3'	218
	R	5'-CAAGACAGTATCTGTCCTCCAGAC-3'	
mBcl2L10	F	5'-TGAGGAAGTCCTCCAGCCTA-3'	240
	R	5'-GCAGCTCAAGAACCCAGAAC-3'	

F, 5'-forward primer-3'; R, 5'-reverse primer-3'

Cell culture, treatment and cell viability assay

The MCF-7, HEK293 or Hela cells were grown in full medium (RPMI 1640 or DMDM, Gibco, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) in 5% CO₂, at 37°C, according to the ATCC recommendation. For estrogen deprivation, MCF-7 cells were washed three times with PBS then further cultured in DMEM with 10% charcoal/dextran-treated FBS (Hyclone, Logan, UT, USA) for 48 h. This time point served as a 0 hour time point control. Cells were either further cultured for 10 h or 50 nM 17 β -estradiol (E2) was added for various times before harvest. To mimic the hypoxia condition, 500 μ M CoCl₂ was applied to 2 \times 10⁵ MCF-7 or HEK293 cells for the indicated times. To study apoptosis, HEK293 cells or the transfectants were incubated with 100 nM staurosporine for 12 h. Viability was determined at various time points by trypan blue exclusion, counting at least 100 cells from each individual culture. The percentage of cell survival was calculated as the number of surviving cells per total cell count [19].

Tissue expression pattern analysis by RT-PCR

To determine the expression patterns of Bok mRNA in different human tissues, PCR was performed using the primers listed in table 1 on a panel of first-strand cDNAs from various human tissues (Origene, Rockville, Md.). The PCR conditions were as described in the manufacturer's protocol with 30 cycles performed on the β -actin control employing Taq polymerase. To examine the expression of members of the Bcl-2 family in mouse, mouse hearts were dissected from two animals. Each dissected mouse heart was divided into two parts and incubated in either 21% O₂ (normoxic condition) or 1% O₂ (hypoxic condition) for 24 h. Total RNA was purified and normalized by determination of concentration at 260 nm. On microgram of RNA was used for reverse transcription. PCR was performed using the primers listed in table 1 with 28 cycles. β -Actin served as a control.

Immunohistochemical investigations

Biopsies were taken following a study protocol approved by the Ethics Committee of the University Hospital in Basel. Briefly, cryostat-frozen sections were fixed in 3% paraformaldehyde followed by application of normal rabbit serum (Vector, Burlingame, Calif.). The slides were then incubated with the primary antibody (1:50 polyclonal rabbit anti-Bok, Sc11424; Santa Cruz, Calif.) overnight at 4°C. After one wash, specimens were blocked with avidin (Zymed, San Francisco, Calif.) then with biotin (Zymed). The sections were incubated with biotinylated goat anti-rabbit IgG antibody (Vector). They were then washed again and incubated with a peroxidase block (Zymed), and reacted with the ABC Elite Kit (Vector). 3,3'-Diaminobenzidine tetrahydrochloride tablets

(Sigma, Deisenhofen, Germany) were added under light protection. The reaction was then stopped with PBS. Sections were counterstained with hemalum, transferred to tap water, dehydrated and mounted in Pertex (Medite, Nunningen, Switzerland).

Cellular fractionation and Western blotting

Cells were pelleted and suspended in 100 μ l of an enzymatic reaction buffer [19], supplemented with protease inhibitors and lysed by sonication for 15 s in an ice bath. The lysate was centrifuged for 10 min at 1000 g for removal of unlysed cells and nuclei. The supernatant was then centrifuged for 1 h at 100,000 g (Kontron Instruments, Munich, Germany). The final supernatant served as a cytosolic fraction, the pellet as a crude membrane fraction which was resuspended in one volume of the lysis buffer with 10% Triton X-100. The protein concentration of both cytosolic and membrane fractions was determined by a BioRad protein assay (BioRad, Hercules, CA, USA).

Alkali extraction of membrane proteins was as described previously [20]. Briefly, the membrane fraction was resuspended (1 mg protein/ml) in freshly prepared 0.1 M Na₂CO₃ (pH 11.5) and incubated for 20 min on ice. The membranes were then pelleted by centrifugation. Membrane proteins corresponding to 20 μ g protein were separated by 15% SDS-PAGE (BioRad) and verified using anti-Bok antibody Sc11424 (Santa Cruz).

To determine oligomerization of hBok, the mitochondrial-enriched fraction was suspended in isotonic HIM buffer [21]. Briefly, bismaleim-ido-hexane (Pierce, Rockford IL, USA) was added to the membrane fraction from a tenfold stock solution (in dimethyl sulfoxide) to a final concentration of 10 mM. After incubation for 30 min at room temperature, the cross-linker was quenched by the addition of 1 M Tris-HCl, pH 7.5, to a final concentration of 20 mM. After quenching, the samples were lysed and analyzed by Western blot with an anti-Bok antibody Sc11424 (Santa Cruz).

Fractionated cell lysates were boiled for 10 min. Samples (20 μ g) were separated on 15% SDS-PAGE, electrotransferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA), immunoblotted with a rabbit polyclonal anti-His antibody (Milan Analytica, Milan, Italy) or anti-Bok antibody (Santa Cruz), and developed with NBT/BCIP (Sigma, St. Louis, Mo.).

Subcellular localization of hBok proteins

To examine the subcellular localization of endogenous hBok, 10⁵ MCF-7 cells were seeded on sterile glass coverslips. After 12 h culture with or without 100 nM staurosporine or 50 nM epothilone B (Novartis, Basel, Switzerland), cells were washed with PBS and fixed with 4% paraformaldehyde (in 10 mM PIPES, 2 mM MgCl₂, 2 mM KCl, 300 mM sucrose and 2 mM EGTA). Cells

were further quenched with 20 mM NH_4Cl and permeabilized in 0.25% Triton X-100. A rabbit anti-Bok antibody (Sc-11424; Santa Cruz) was used as primary antibody at 1:200 dilution. The secondary antibody was Alexa-488-conjugated goat anti-rabbit IgG at 1:1000 dilution. To examine the subcellular localization of transfected hBok, HEK293 or Hela cells were transfected with 0.3 μg of expression plasmids encoding GFP-hBok using Effectene reagent (Qiagen, Hilden, Germany). Cells were incubated with 100 nM Mitotracker Red 580 (Molecular Probes,

Eugene, Ore.) and then fixed in 3.7% formaldehyde 24 h post-transfection. The results were further confirmed by a rabbit polyclonal antibody against Bok BH3 domain (AP1310a; Abgent, San Diego, Calif.). Images were acquired with a confocal microscope (Leica NT SP1; Leica, Heidelberg, Germany) with oil immersion using a $\times 63$ lens on a Leica DM RxE. The images were acquired with Leica confocal software with the image format of 1024×1024 pixels \times 8 bit, and transferred to a graphics program for printing.

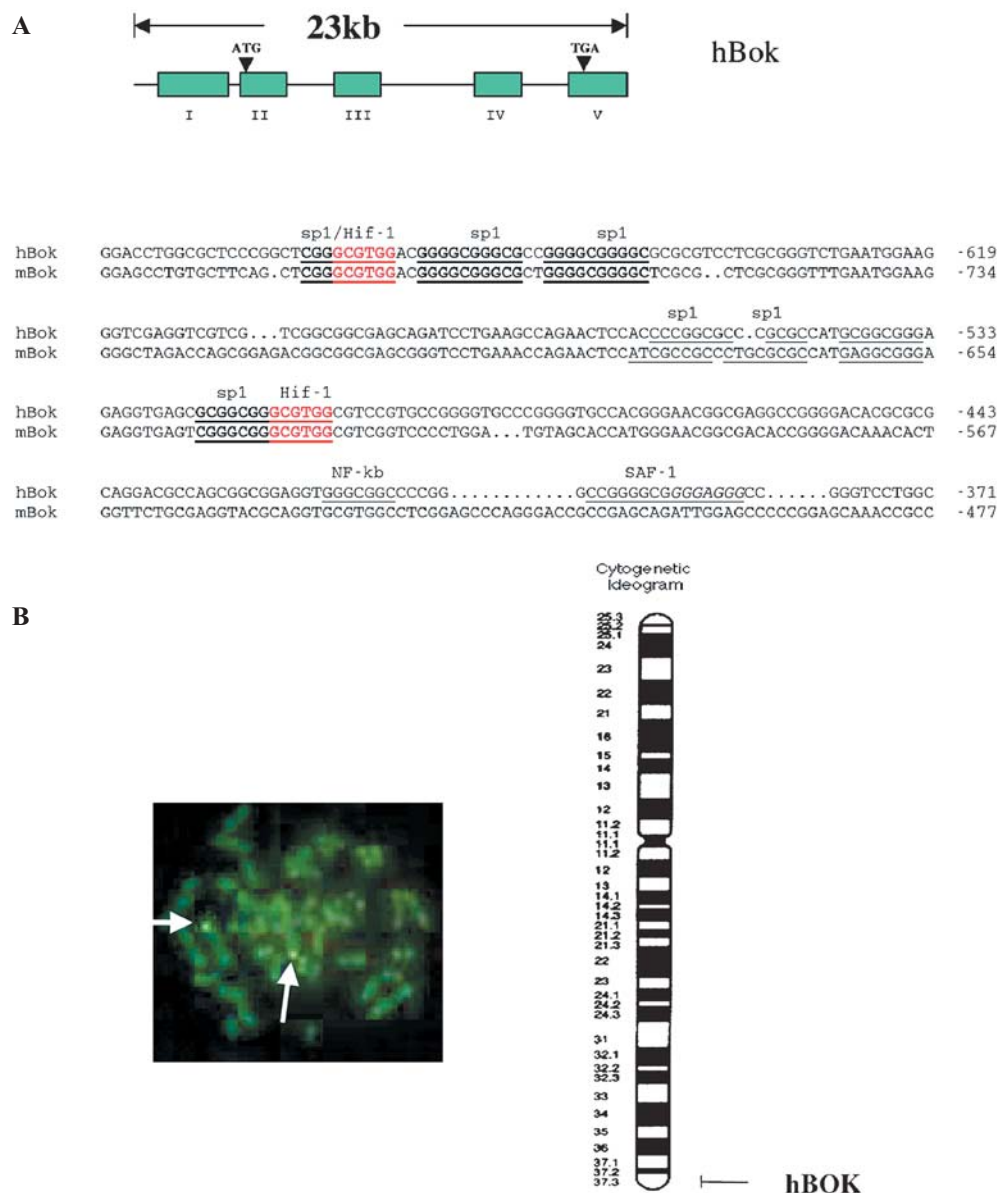


Figure 1. Genomic organization and chromosomal localization of the human *Bok* gene (*hBok*). (A) Schematic representation of the *hBok* gene structure. The boxes represent the exons. The exon numbers are indicated below the boxes. The thin lines denote introns and flanking regions. The lower panel shows the alignment of the conserved 5'-UTR sequences of human (-371 to -709) and mouse (-477 to -821) *Bok* genes. Underlines represent transcriptional factor-binding sites. (B) Left panel shows that in situ hybridization of a biotin-labeled human *Bok* probe to human metaphase cells resulted in specific labeling on chromosome 2 (arrow). Right panel shows results of FISH mapping. The detailed position of the *Bok* gene, on human chromosome 2q37.3, was determined from careful analysis of 40 metaphase cells and compared to the NCBI STS database.

Co-immunoprecipitation analysis

HEK293 cells (10^6) were transfected with 1 μ g of the expression plasmids pcDNA3-his-tBid, pcDNA3-his-Bnip3 and pc53-SN3 encoding Bid, Bnip3 and p53, respectively. Twenty-four hours after transfection, cells were lysed in M-PER protein extraction reagent (Pierce). His-Bind resin (50 μ l; Novagen, San Diego, CA, USA) or 5 μ g of anti-p53 (Ab-6) antibody (Oncogene, Cambridge, MA, USA) were incubated with 100 μ l cell lysate. Protein G-fast Flow Sepharose (25 μ l; Amersham, Uppsala, Sweden) was added to collect the anti-p53 antibody. The gels were collected after washing and boiled in SDS sample buffer followed by SDS-PAGE and Western blot with anti-Bok antibody (Sc-11424, Santa Cruz).

Results

Isolation and characterization of the human Bok gene (hBok)

To identify the chromosomal location of the *Bok* gene, we isolated and identified one positive PAC clone, which covers all coding regions of the human *Bok* gene from a human P1 genomic library (see Materials and methods). Sequence analysis revealed that it contains four coding exons in which the exon-intron organization is conserved between human, mouse and rat genes, but the intron sizes are different between these species (fig. 1A). The complete *Bok* gene spanned about 23 kb of genomic DNA. We then used this PAC clone as a probe for FISH studies. We examined chromosomes in metaphase from a normal male for fluorescence signals. Out of 40 metaphases examined, 32 possessed a specific signal in the region 2q37 (fig. 1B). No background signals were observed. Localization to 2q37 is consistent with in silico analysis of the draft human DNA sequence at the National Center for Biotechnology Information, which mapped the genomic locus of *hBok* in human chromosome 2 between the markers D2S125 and chr2_qTEL. Together with all other data, the *Bok* gene was localized on human chromosome 2q37.3 (fig. 1B, right panel).

Differential expression of hBok in human tissues

Semiquantitative RT-PCR analysis (semi-Q-RT-PCR) was performed using a pair of primers corresponding to sequences flanking the first intron (Materials and methods). As shown in figure 2A, human *Bok* was expressed mainly in colon, stomach, testis, placenta, pancreas, ovary and uterus. The expression in fetal liver exhibited a higher level than that in adult tissue. The expression of the human *Bok* protein in various human tissues was also examined with immunohistochemistry. Figure 2B shows the localization of hBok protein in ovarian and testicular tissue samples. Human *Bok* was expressed predominantly

in the granulosa cell compartment of ovarian follicles, consistent with the observation for rBok [4], and in the spermatocytes of testicular seminiferous tubules.

The expression was further examined in various hormonally regulated cancer cell lines using semi-Q-RT-PCR. All seven breast cancer cell lines displayed much lower *Bok* expression than HBL100, which is considered a non-metastatic breast epithelial cell line. In contrast, granulosa cell tumors lacked mRNA expression. However, clearcut expression of *Bok* mRNA was detected in some other

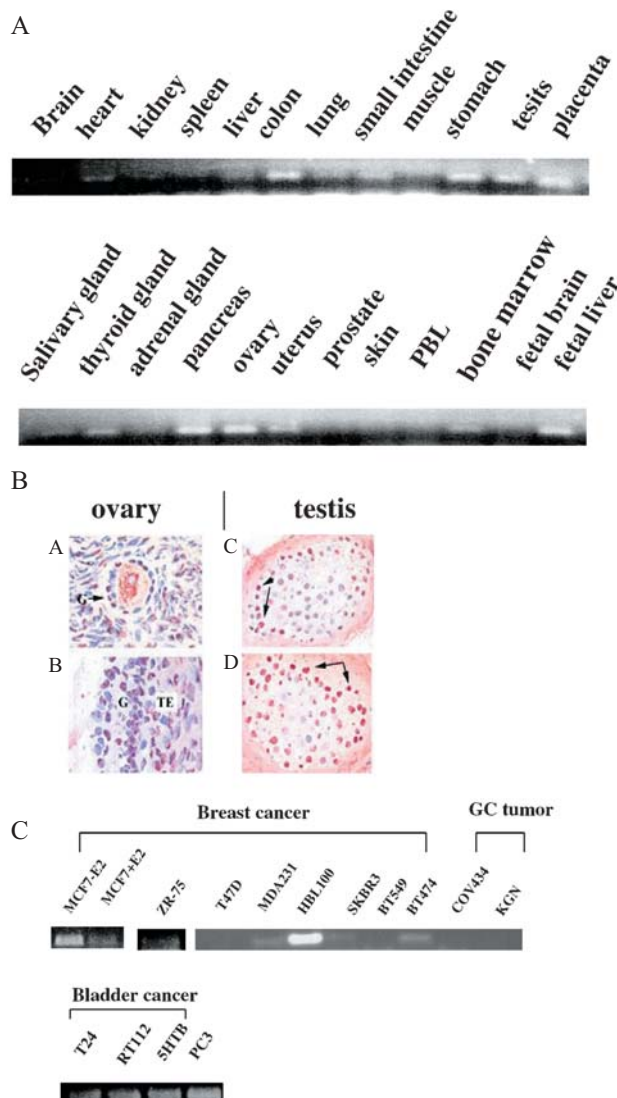


Figure 2. Expression of *hBok* mRNA species varies in different tissues. (A) PCR was performed on a panel of first-strand cDNAs prepared from different human tissues (Origene). The PCR products obtained from each reaction following 30 cycles were separated on a 1.2% agarose gel and visualized by SYBR green I. (B) The localization of hBok protein was examined with immunohistochemistry in ovarian follicles (A, B) and testicular seminiferous tubules (C, D). G, granulosa cells; TE, theca cells; arrows, spermatocytes. (C). *hBok* expression was examined in various cancer cell lines using semi-Q-RT-PCR.

cancer cells, such as bladder and prostate cancer cells (fig. 2C).

Differential regulation of *hBok* expression

During our initial studies on the regulation of apoptosis in hormonally regulated breast cancer cell lines, we observed that hBok expression was enhanced in MCF-7 cells both by E2 withdrawal and by hypoxia. MCF-7 cells showed low but detectable levels of *hBok* mRNA. Cells cultured in an estrogen-free, serum-free medium for 2 days or treated with the steroidal anti-estrogen, ICI 182780, generated increased levels of *hBok* mRNA, whereas E2 treatment induced the downregulation of *hBok* mRNA (fig. 3A). In contrast, c-Myc expression was increased by addition of E2 (data not shown). Furthermore, the expression in MCF-7 cells treated with various concentrations of CoCl_2 , a condition which was chosen to mimic hypoxia, resulted in increased *hBok* expression after 24 h. However, combined treatment of ICI 182780 and hypoxia did not further enhance the expression of *hBok*. Similar observations were made with HEK293 cells (data not shown). Although staurosporine induced apoptosis in both cell lines, expression of hBok was not enhanced (fig. 3B), indicating that expression of hBok is regulated distinctively upon apoptotic stimuli.

Hypoxia has been shown previously to induce brain injury and apoptosis of cardiomyocytes, resulting in ischemia, and this process is, at least in part, preventable by estrogen [22, 23]. We therefore further examined the expression of

Bok in mouse hearts. As demonstrated in figure 3C, *Bok* was expressed at very low levels in primary cells, but hypoxia caused a significant increment in *Bok* mRNA level after 24 h. The expression level of Bnip3 was also observed to be increased, as Bnip3 has already been reported as a hypoxia-response gene during hypoxia-induced apoptosis [24]. The expression of three other BH3-only genes was also examined under the same condition: the expression of *Bad* was decreased, whereas that of *Noxa* [25] and *Bmf* [26] remained unexpressed and that of *Bcl2L10* [27, 28] increased (fig. 3D). Thus, hypoxia appears to regulate expression of *Bok* both in the human and mouse. Examination of the *hBok* promoter region revealed the existence of two conserved Hif-1 α binding sites (fig. 1A). These results suggest that the expression of *Bok* may be specifically hypoxia regulated in certain organs.

Death signals alter the subcellular localization of hBok

We first assayed localization of hBok by subcellular fractionation studies. Cells were lysed and fractionated into a cytosolic and a membrane fraction after removal of the low-speed nuclear pellet. As shown in figure 4A, the endogenous hBok protein could be detected in both cytosolic and membrane fractions upon subcellular fractionation of healthy MCF-7, whereas Bax was detected mainly in the cytosolic fraction [29]. Similar results were obtained in HEK293 and Hela cells lysed in a buffer with

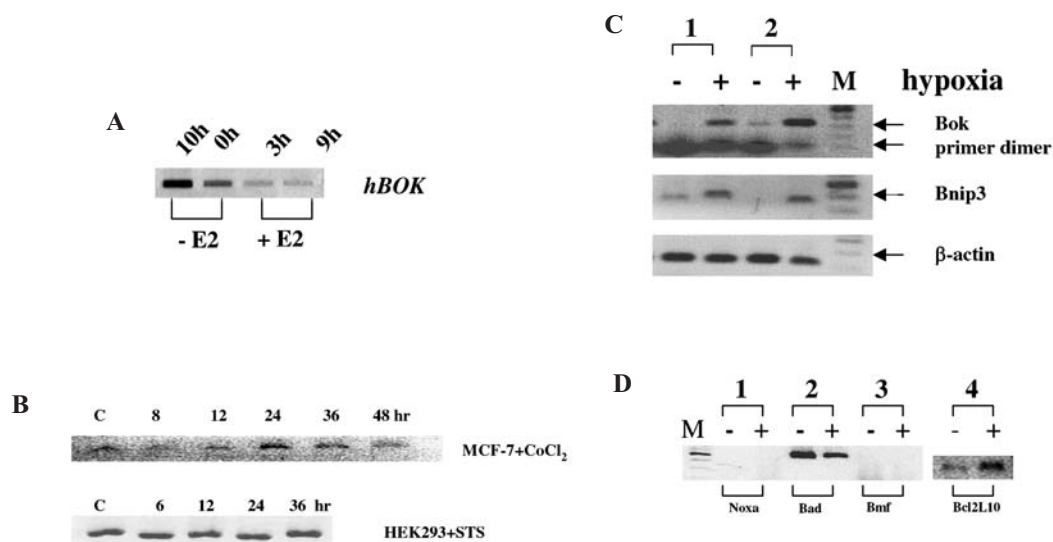


Figure 3. Regulation of *hBok* mRNA expression in various conditions studied by semi-Q-RT-PCR. (A) MCF-7 cells were cultured in an estrogen (E2)-free, serum-free medium for 48 h. 10 h indicates further culture for 10 h in the estrogen-free medium (-E2) or addition of 50 nM E2 for 3 or 9 h. (B) Expression of *hBok* mRNA was examined in 2×10^5 MCF7 cells treated with 500 μM CoCl_2 for the indicated times. Expression of *hBok* mRNA was also examined in HEK293 cells after treatment with 100 nM staurosporine (STS). (C) Examination of *Bok* expression in dissected mouse hearts from two animals. Each dissected mouse heart was divided into two parts and incubated in either 21% O_2 condition (-, normoxia) or 1% O_2 (+, hypoxia). Total RNA was normalized by determination of concentration at 260 nm. One microgram of RNA was used for reverse transcription. As controls, the expression of *Bnip3* and the housekeeping gene β -actin were examined in parallel. (D) Expression of four members of the Bcl2 family, *Bad*, *Noxa*, *Bmf* and *Bcl2L10* were examined in the same condition as in C.

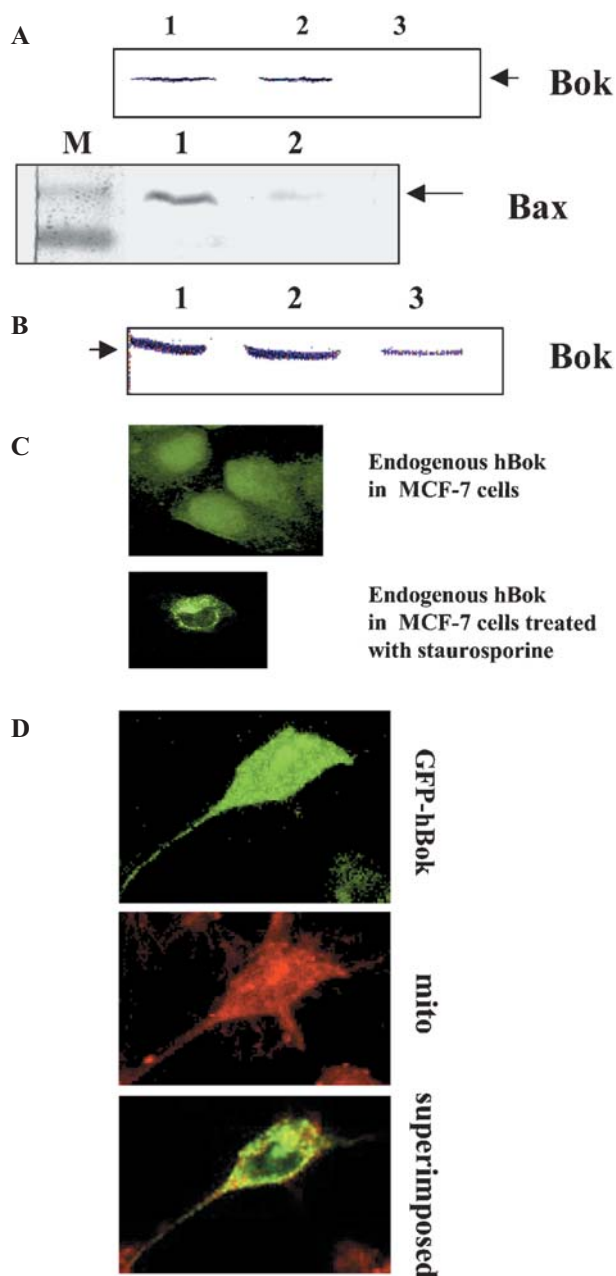


Figure 4. Intracellular localization of hBok. (A) Subcellular localization of Bok by cell fractionation. 5×10^6 MCF-7 cells were collected, lysed and fractionated for high-speed cytosolic and membrane fractions as described in Materials and methods. The protein samples were separated by 15% SDS-PAGE and analyzed by Western blot with a polyclonal antibody against human Bok (Santa Cruz) or Bax (BD/Pharmingen). The proteins were fractionated into cytosolic (1) and membrane (2) fractions. 3 indicates alkaline-treated membrane fractions. (B) MCF-7 cells were treated with 100 nM staurosporine for 12 h. Cell lysates were prepared and analysed as in A. (C) Immunolocalization of endogenous hBok in MCF-7 cells treated with or without 100 nM staurosporine. Micrograph showing a single, confocal image plane of MCF-7 cells labeled with a rabbit anti-Bok antibody (Sc-11424) followed by an Alexa-Fluor-488-conjugated secondary antibody (green). (D) Subcellular localization of GFP-Bok by confocal microscopy with a magnification of $\times 63$. HeLa cells were transiently transfected at GFP-Bok as described in Materials and methods. MitoTracker was used to label mitochondria (mito).

or without detergent (data not shown). Moreover, an alkali treatment was effective in extracting hBok from the membrane, indicating that in healthy cells, Bok appears to associate only weakly with the membranes (fig. 4A). However, after treatment of the cells with various apoptotic stimuli, the membrane-bound hBok could not be released by alkali treatment, indicating that hBok had become integrated into the membrane (fig. 4B). Furthermore, we assessed the subcellular localization of hBok by confocal laser scanning microscopy. In MCF-7 cells, the expression of endogenous hBok was rather diffuse throughout the cell, examined using two different anti-Bok antibodies (fig. 4C). However, the distribution of hBok became localized together with the membrane when cells were treated with staurosporine (fig. 4C) or epothilone B (not shown). Moreover, the full-length hBok was fused to a GFP expression vector. As shown in figure 4D, the experiment of transient overexpression of hBok in HeLa cells revealed that hBok was mainly associated with mitochondria, as it was localized very strongly with the mitochondrial marker. Similar results were found in HEK293 cells. These results suggest that in healthy cells, hBok is associated loosely with the membrane in a hit-and-run mode. The insertion and accumulation of hBok on membranes is then enhanced through the activity of death signals, resulting in the integration of the membrane-bound hBok protein into the membrane.

Oligomerization of hBok is promoted by apoptotic stimuli

Bnip3 and p53 were induced or accumulated together with hBok during hypoxia (fig. 3) [24] and because Bnip3, p53 and hBok share several properties, such as their common cytoplasmic and mitochondrial localization and their pro-apoptotic function, we explored the possibility of a functional co-operation of these proteins during apoptosis. Similar to the staurosporine treatment, co-expression of Bid [20] and Bnip3 further sensitized cells to apoptosis, which could be counteracted by expression of BFL-1 (data not shown). Furthermore, oligomerization of hBok was detected in all transfectants with Bnip3, Bid and p53, indicating that these BH3-only proteins were able to induce oligomerization of hBok, as seen with staurosporine treatment (fig. 5A). However, p53 was not able to further increase the level of apoptosis under hBok overexpression (data not shown). Next, we explored the mode of action of Bnip3, Bid and p53 by examining their interaction with the endogenous Bok by co-immunoprecipitation. HEK293 cells were transfected with His-Bnip3, p53 or His-Bid (Materials and methods). Cellular lysates of transfectants were incubated with anti-His or anti-p53 antibodies for pull-down assay. hBok was then detected by anti-Bok antibody. As shown in figure 5B, hBok could only be detected in Bnip3 interaction. However, p53 and Bid could not be detected. These results indicate that hBok

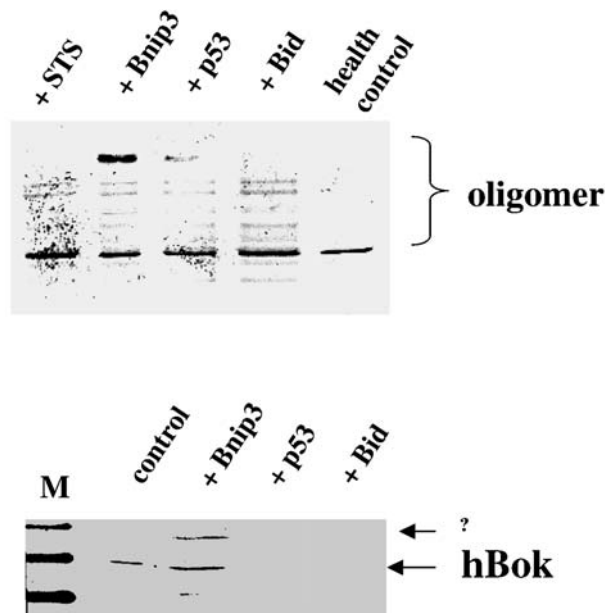


Figure 5. Oligomerization of hBok by BH3-like proteins and interaction of hBok with Bnip3. (A) Oligomerization of hBok. The membrane fraction of cell lysates was incubated with 10 mM BMH cross-linker for 30 min at room temperature. Mitochondrial proteins were extracted with a Chaps buffer and subjected to SDS-PAGE under non-denaturing conditions. Cells were transfected without (health control) or with the genes indicated or treated with 100 nM staurosporine (STS). Western blot was performed with a rabbit anti-Bok antibody (Sc-11424, Santa Cruz). (B) HEK293 cells were transfected with Bnip3, p53 or tBid with non-transfected cells as a control. Western blot was performed with a polyclonal antibody against human Bok (Sc-11424, Santa Cruz).

interacts with Bnip3 but not with p53 or Bid directly, suggesting that regulation of hBok activation is mediated, at least in part, by two distinct mechanisms.

Discussion

The present studies demonstrate that the function of Bok during apoptosis is regulated both at the transcriptional and at the post-transcriptional level. In both rat and mouse, the expression of Bok is almost entirely restricted to reproductive tissues [4]. Although human Bok is expressed in human ovarian granulosa cells and has been reported to be expressed in pronucleate oocytes [30], expression of human *hBok* mRNA was detected outside the reproductive organs and found in many tissues, suggesting that human Bok may play a more important role in homeostasis and development than in the rat or mouse. Indeed, expression is higher in fetal than in adult liver, suggesting a functional role for hBok in the function of that organ during fetal development.

We also demonstrated that the expression of hBok is regulated by only certain apoptotic stimuli, such as estrogen withdrawal and hypoxia. In accordance with our observa-

tions, a recent study showed that DNA damage induces expression of Bok in neuroblastoma cells [31]. However, the treatment of breast cancer cells with staurosporine was not able to stimulate the expression of Bok, indicating that regulation of Bok expression may vary according to the type of apoptotic signal. Sequence analysis on promoter regions of the human, mouse and rat *Bok* gene revealed a highly conserved region which contains two Hif-1 α -binding sites. Further experiments are underway to investigate the function of Hif-1 α in the regulation of Bok. How does the induction of Bok during estrogen deprivation and hypoxia relate to the initiation of apoptosis? Bik, a BH3-only protein, could be induced by estrogen deprivation [32]. Bnip3, another BH3-only protein, is also induced by hypoxia [24]. One possibility for the initiation of apoptosis is that activation of Bok is triggered by distinct BH3-only proteins, such as Bnip3 or Bik. Knock-down experiments by short interfering RNAs (siRNA) to Bok will facilitate analysis of the role Bok plays in the apoptotic pathway triggered by estrogen deprivation and hypoxia.

Like many members of the Bcl-2 family, hBok possesses a hydrophobic COOH-terminal domain. hBok can be readily isolated from both the membrane and the cytosolic fraction in healthy cells and it could also be released by alkali treatment, indicating that it is not an integral membrane protein, which distinguishes it from Bcl-x_L and Bak, which are [33], or Bax, which resides primarily in the cytoplasm of healthy cells [25, 34]. The hydrophobic COOH-terminal residues of Bax and Bcl-w form a helix that creates a hydrophobic groove, into which a BH3 domain may bind [35, 36]. A domain that encompasses the hydrophobic helices H6 and H7 of Bid (a cytosolic protein that lacks the hydrophobic C-terminal domain) is the minimal region necessary for strong mitochondrial-binding activity [37, 38]. Furthermore, the acidic phospholipid cardiolipin is the receptor for Bid [39]. All these observations lead us to speculate that there is a domain beside the COOH terminus responsible for the weak membrane association of hBok. Further study should investigate this issue.

The weak association of hBok with the mitochondrial membranes of healthy cells is dramatically altered in dying cells, where hBok becomes firmly integrated into the membrane. Several studies have shown that damage signals induce the expression of a BH3 domain-only protein, which then replaces the hydrophobic COOH-terminal helix, causing the translocation of Bax from the cytosol to mitochondrial membranes, where it forms oligomers [8, 11, 40, 41].

Interestingly, our co-immunoprecipitation studies could only detect the interaction of hBok with His-Bnip3 but not with p53 and His-Bid, suggesting that the regulation of hBok activation is mediated, at least in part, by two different mechanisms: oligomerization of hBok can be

triggered directly by Bnip3 but oligomers formed only in the presence of p53 and Bid via a specific but still unknown element. Hypoxia-induced activation of hBok, Bnip3 and p53 and oligomerization of hBok could be triggered by Bnip3 and p53, which suggests that these factors act together. Bnip3 is a BH3-only protein, and regulates activation-induced cell death of effector cytotoxic T lymphocytes [42]. Bnip3 has previously been demonstrated to interact with Bcl-2/Bcl-xL [43] and CD47 [44]. hBok could be another Bnip3-interacting protein; the binding activates hBok by triggering its oligomerization and is thus associated with the initiation of cell death. Overexpression of Bnip3 alone has been reported to initiate permeability transition pore opening and induce a necrosis-like cell death [45]. However, many cellular events, such as hypoxia, are accompanied by changes in the expression of more than one gene, which may work co-operatively. Whether the association of Bnip3 with hBok plays a role in mediating necrotic cell death remains to be elucidated.

p53 is a key tumor suppressor, which induces cell cycle arrest, DNA repair and apoptosis. p53 is normally thought to be localized in the nucleus and to regulate numerous p53-responsive genes [46, 47]. However, recent studies revealed that p53 also has a role in the cytoplasm and functions as a BH3-like protein during apoptosis [15, 16]. Our studies showed that although p53 was unable to interact directly with hBok, it was able to activate hBok by triggering oligomerization. Many pro-apoptotic Bcl-2-related genes, such as Bax, DR5, Bid, Puma and Noxa, are p53-dependent target genes [48–51]. hBok has not been shown to be a direct target of p53, and we could not exclude some function of nuclear p53 in this study. Unlike direct p53-Bak interaction [15], p53 could not interact with hBok directly. p53 may sequester Bcl-2/Bcl-xL to release pro-apoptotic Bcl-2 proteins or BH3-only proteins, such as Bnip3, as suggested with Bax [16].

At present, our data suggest that the weak binding to the mitochondrial membrane represents a primary contact in preparation for activation of apoptosis. Interaction of BH3 domain proteins, such as Bnip3, with hBok induces a secondary membrane insertion step resulting in integration of hBok into the membrane.

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