Identification of a caspase 3-independent role of pro-apoptotic factor Bak in TNF-α-induced apoptosis

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Abstract **By using our recently developed gene discovery system, we have identified Bak, a member of the Bcl-2 family, as a pro-apoptotic factor in the tumor necrosis factor (TNF)-α-induced apoptotic pathway in caspase 3-deficient cells. Unlike Bcl-2, Bak stimulates several apoptotic pathways, however the molecular mechanism(s) of its action remains unclear. For example, it is unclear whether Bak induces apoptosis in caspase 3-deficient cells. In this study, we examined the effects of overexpression of Bak in MCF-7 cells that lack caspase 3. We found that despite the absence of caspase 3 in MCF-7 cells, they were more sensitive to the cell death effects of Bak as compared to caspase 3-expressing HeLa S3 cells. The targeting of Bak function by ribozymes suggests that Bak is required for the TNF-α-induced apoptotic pathway in caspase 3-deficient cells. This study demonstrates the caspase 3-independent function of Bak in the TNF-α-induced apoptotic pathway.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Hammerhead ribozyme; Bak; Caspase 3; Apoptosis; Ribozyme library

1. Introduction

To improve the efficiency of ribozymes for cleaving their target RNAs in vivo, we initially constructed ribozymes with better accessibility and specificity to the target sites [1–8]. This was obtained by combining the cleavage activity of a hammerhead ribozyme with the unwinding activity of an endogenous RNA helicase(s) that was recruited by a poly(A) motif linked to the ribozyme ($tRNA^{Val}$-Rz-A60) [9–11]. We demonstrated that such a novel poly(A)-connected hybrid ribozyme has substrate-unwinding activity and stronger cleavage activity. We then constructed a randomized ribozyme library and demonstrated its use for the identification of genes by a loss of phenotype approach [9–12]. Sequencing of transfected ribozyme from cells that lose a selected phenotype led to the rapid identification of genes involved in that phenotype. We employed this rapid gene discovery protocol to identify genes involved in tumor necrosis factor (TNF)-α-induced apoptosis in caspase 3-deficient MCF-7 cells and identified Bak as one of the genes [11]. The Bcl-2 family of proteins plays a central role in the regulation of programmed cell death (apoptosis), which is induced by a wide variety of stimuli such as serum deprivation, the FAS antigen and TNF-α [14–18]. The pro-apoptotic protein Bak, an antagonist of Bcl-2 and its homologs, has been cloned and identified as an E1B19K-binding protein. Since E1B19K is known to inhibit apoptosis, it has been suggested that the E1B19K protein functions, at least in part, by binding to the pro-apoptotic Bak protein and inhibiting its pro-apoptotic function. Recent studies have revealed that overexpression of Bak causes apoptosis of cancer cells [19–21]. However, the molecular basis of the actions of Bak remains unclear. It has been proposed that Bak functions in a caspase 3-dependent manner [13], however we have identified Bak as a mediator of the TNF-α-induced apoptosis pathway in MCF-7 cells which lack caspase 3 [11]. We have now characterized the role of Bak in caspase 3-deficient (MCF-7) and expressing (HeLa S3) cells by its exogenous expression and targeting by hammerhead ribozymes. Our study demonstrates that in addition to its previously described caspase 3-dependent function [13], Bak has a caspase 3-independent role in TNF-α-induced apoptosis.

2. Materials and methods

2.1. Construction of Bak-expressing and -targeting vectors

Human Bak cDNA was cloned from a cDNA library derived from human heart (TaKaRa, Tokyo, Japan) by PCR amplification. Primers were based on the published DNA sequence [19] as follows: Bak sense 5′-GAA TTC ATG GCT TCG GGG CAA GGC CCA-3′ and antisense 5′-GGA TCC TCA TGA TTT GAA GAA TCT TCG T-3′. Sequences of the PCR-amplified Bak fragment were confirmed by direct sequencing. The desired cDNA fragment was cloned into the *Not*I and *Bam*HI sites of pEGFP-c2 and the *Eco*RI and *Bam*HI sites of pIRESpuro (Clontech, CA, USA) respectively. The Bak-targeting hammerhead ribozyme plasmid was constructed as described previously [5,8]. All plasmids were confirmed for their sequence integrity by direct sequencing.

2.2. Cell culture, transfection and microscopy

MCF-7 human breast carcinoma cells were a gift from Dr. Wang (University of South Florida College of Medicine, USA). Cells were maintained routinely in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 μM non-essential amino acids and 1 mM sodium pyruvate (Gibco BRL, MD, USA) in an atmosphere of 5% CO_2 in air at 37°C. HeLa S3 cells (RIKEN gene bank, Wako, Japan: no. RCB0191) were also maintained in MEM plus 10% fetal bovine serum. Cells were transfected with the cationic transfection reagent Trans-IT LT1 (Mirus, WI, USA) following the manufacturer's instructions. Transiently transfected cells were typically assayed after 24 h. Phase-contrast and fluorescence images were recorded with an LSM510-V2.01 system (Carl Zeiss, Jena, Germany).

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2.3. Preparation and analysis of cell extracts

Protein lysates were prepared by treating monolayers of cells with lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40) supplemented with a protease-inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland). The concentration of proteins in lysates was determined by a standard dye-binding assay (Bio-Rad Protein Assay; Bio-Rad, CA, USA). 50 μg aliquots of total protein were resolved on a 10% SDS–polyacrylamide gel and transferred to a PVDF membrane. Immunoblotting was performed by standard methods using a polyclonal anti-Bak antibody (#sc-832; Santa Cruz), a polyclonal anti-actin antibody (#sc-7210; Santa Cruz) and anti-green fluorescence protein (GFP) antibody (a gift from Dr. Nagasaki, AIST, Tsukuba, Japan).

2.4. Analysis by reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared from transfected cells at 70–80% confluency by using the Isogen reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 2 μg total RNA and M-MLV reverse transcriptase (Promega, WI, USA). PCR was performed by using the following gene and ribozyme specific primers. Bak sense 5′-GAA TTC ATG GCT TCG GGG CAA GGC CCA-3′ and antisense 5′-GGA TCC TCA TGA TTT GAA GAA TCT TCG T-3′; β-actin sense 5′-GCA CGG CAT CGT CAC CAA CT-3′ and antisense 5′-AGG GCT GGA AGA GTG CCT CA-3′; DHFR dihydrofolate reductase sense 5′-ATG GTT GGT TCG CTA AAC TGC A-3′ and antisense 5′-CTG GGT ATT CTG GCA GAA GT-3′; caspase 3 sense 5′-ATG GAG AAC ACT GAA AAC TCA GT-3′ and antisense 5′-TTA GTG ATA AAA ATA GAG TTC TT-3′; and Bak-ribozymes sense 5′-TCC CCG GTT CGA AAC CGG GCA CTA C-3′ and antisense 5′-AGA AAA AAA GAT ATC CGG GGT ACC-3′. The amplified DNA fragments were examined by gel electrophoresis and confirmed for the expected size of amplified gene fragments.

2.5. Assay of TNF-α-induced apoptosis

Cells were seeded in six-well plates and treated with mouse recombinant TNF-α (100 ng/ml; Sigma, MD, USA) and cycloheximide (CHX; 5 μg/ml; Sigma, MD, USA) for 24 h. Cells were washed twice with phosphate-buffered saline and harvested with trypsin-EDTA (1–0.5 mM). Cells in each well were counted. The ratio of surviving to dead cells was estimated by comparing the number of cells with TNF-α treatment to that of untreated batches. Three independent experiments were performed.

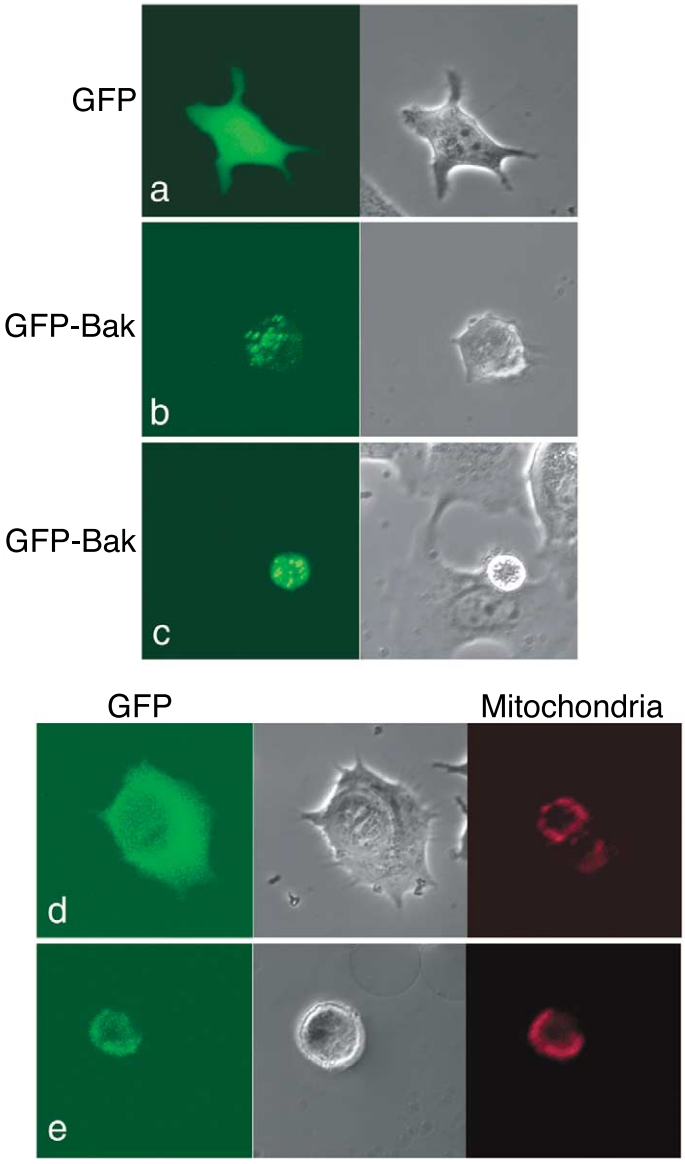

Fig. 1. Effects of expression of GFP-Bak in MCF-7 cells. a: Cells that expressed GFP, as a control, remain alive. b and c: Cells that expressed GFP-Bak died, whereas cells that did not emit green fluorescence (namely cells that did not express GFP-Bak) were alive (c). d: Cells in which DsRed1-Mito was localized in mitochondria. e: Cells that co-expressed DsRed1-Mito and GFP-Bak.

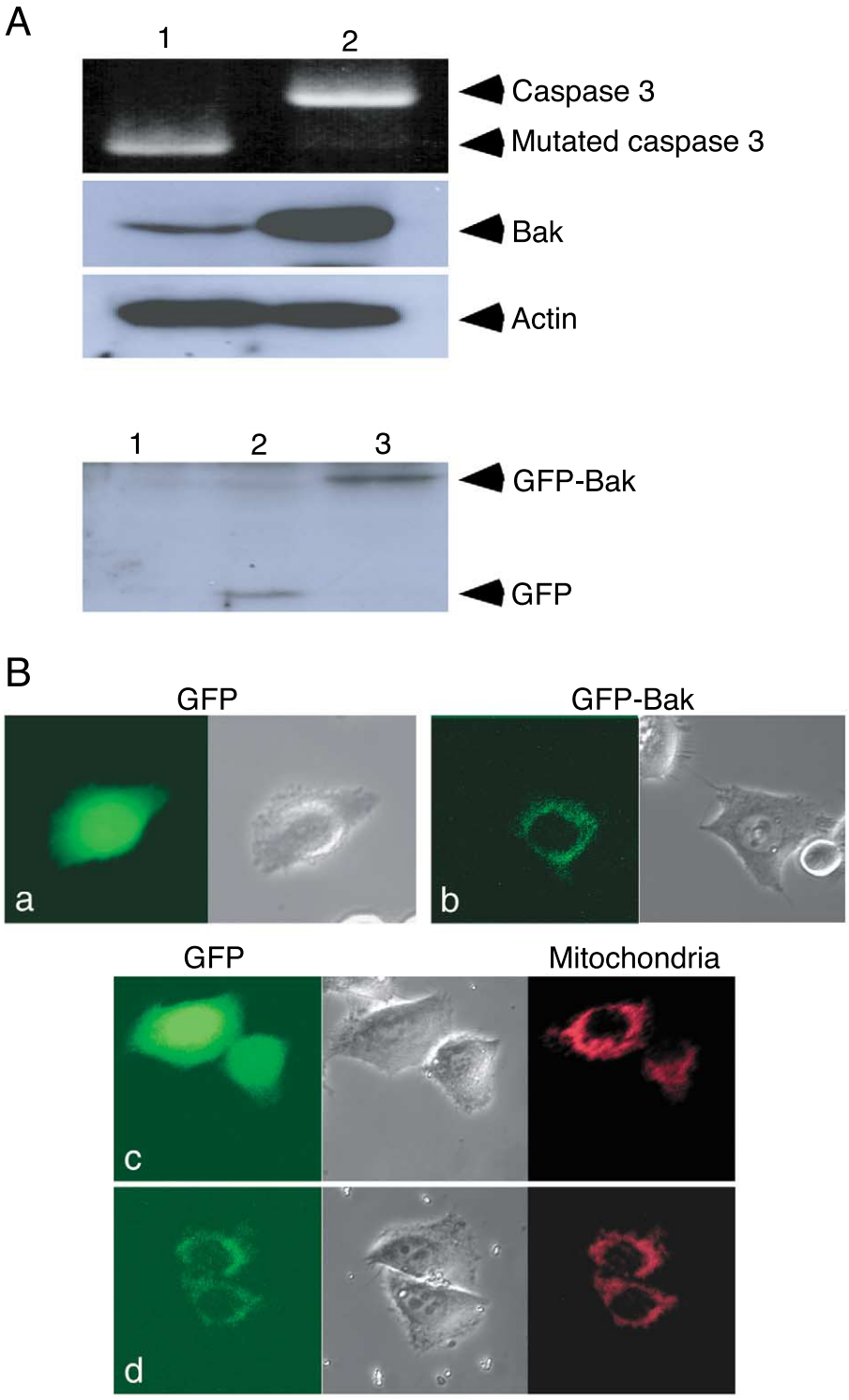

Fig. 2. Expression levels and their effects of Bak and GFP-Bak in MCF-7 and HeLa S3 cells. A: RT-PCR and immunoblotting analyses for the expression of caspase 3, Bak and GFP in MCF-7 and HeLa S3 cells. Upper panel: the caspase 3 mRNA in MCF-7 cells (lane 1) was shorter than that in HeLa S3 cells (lane 2). This deletion results in abrogation of translation of the mRNA. Middle panel: comparison of endogenous Bak expression between MCF-7 and HeLa S3 cells. The levels of Bak in MCF-7 cells (lane 1) and HeLa S3 cells (lane 2) were clearly different. Expression levels of actin are also shown as an internal control. Lower panel: cell lysates were analyzed 24 h after transfections of GFP expression vector (lane 2) or GFP-Bak expression vector (lane 3) or Mock (lane 1). B: In HeLa S3 cells, GFP-Bak did not cause cell death in a transient transfection assay. a: Cells that expressed GFP as a control and (b) cells that express GFP-Bak. c: GFP and DsRed1-Mito proteins were localized to mitochondria. d: DsRed1-Mito and GFP-Bak were colocalized in mitochondria.

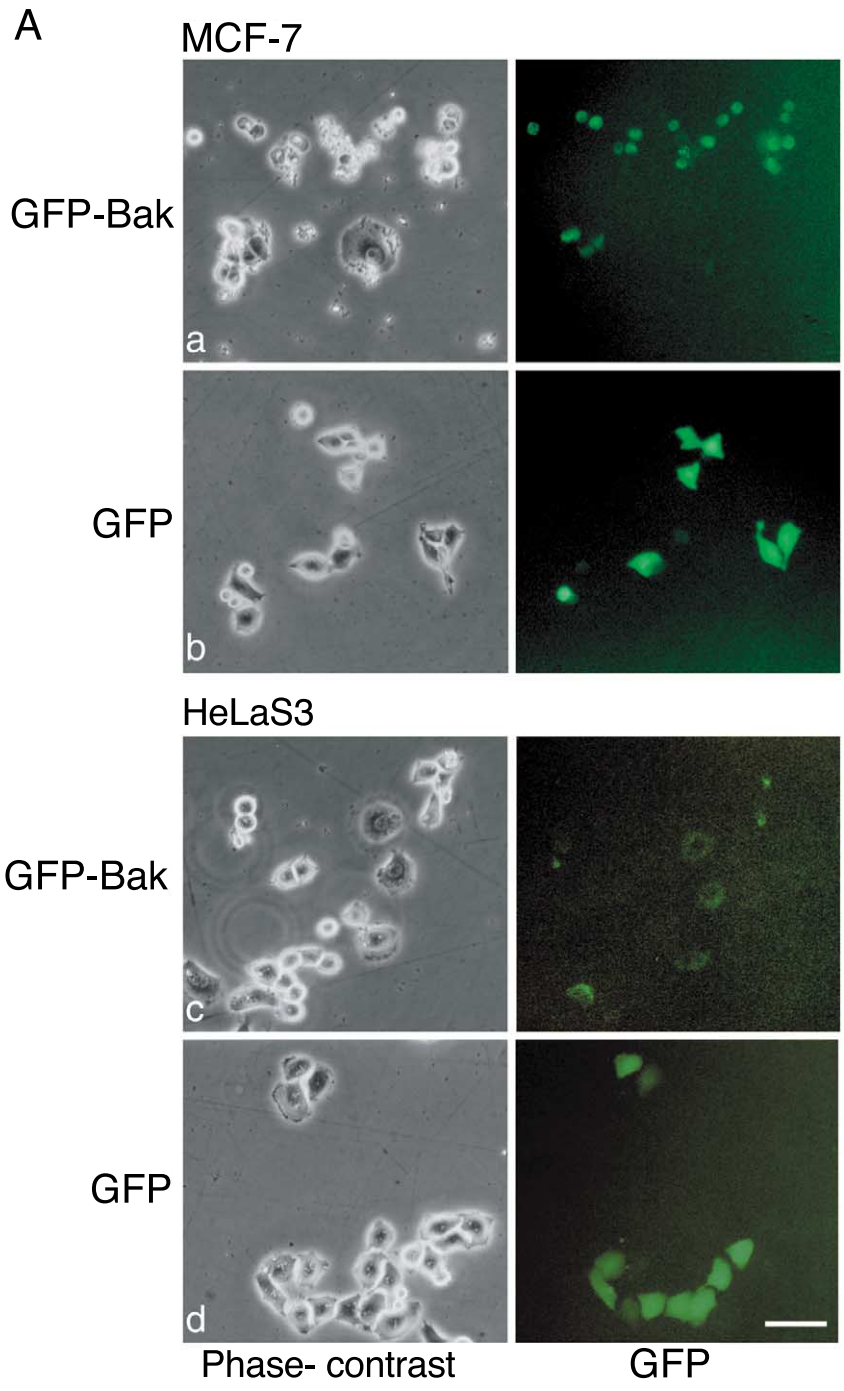

Fig. 3. Cell death effect of Bak. A: MCF-7 cells that expressed GFP-Bak were dead (a). By contrast, most MCF-7 cells that expressed GFP (b), HeLa S3 cells that expressed GFP-Bak (c), or GFP (d) were alive. B: Transfection efficiency was monitored by green fluorescence. Cell death was examined in terms of changes in cell morphology and trypan blue staining. Data are presented as the mean ± S.D. of results of three independent experiments performed in triplicate. Scale bar indicates 50 μm.

3. Results and discussion

3.1. Overexpression of Bak in MCF-7 cells

We examined the effects of the expression of Bak using an expression plasmid that encoded a GFP-Bak fusion protein. When MCF-7 cells (a line of human breast carcinoma) were transfected with plasmid DNA encoding GFP-Bak, the cells died, but no cell death was observed with the expression of GFP alone (Fig. 1). Most noticeably, the untransfected cells (lacking green fluorescence) remained viable in the mix culture of transfected and untransfected cells (Fig. 1c, a dead fluorescent cell, left, is accompanied by a living non-fluorescent cell, right). From these data, Bak appeared to be toxic to MCF-7 cells. These cells have lost caspase 3 owing to a 47 bp deletion within exon 3 of the gene (Fig. 2A, upper panel) [22]. These results suggested that Bak may function in the absence of caspase 3, an important mediator of apoptosis, by a caspase 3-independent pathway.

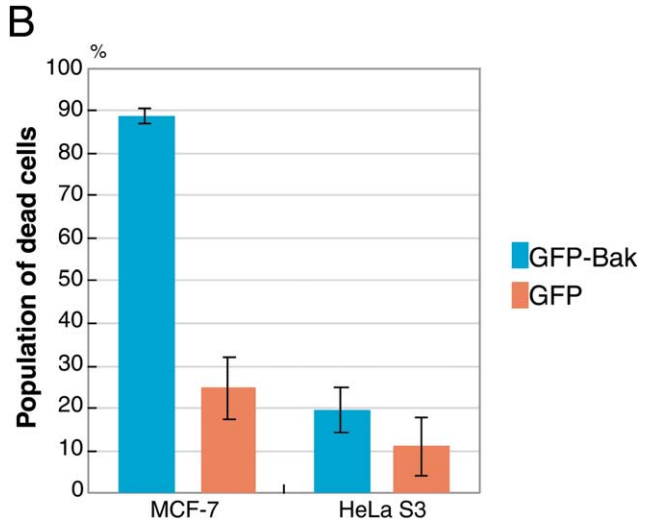

Fig. 3 (*Continued*).

3.2. Overexpression of Bak in HeLa S3 cells

We next examined the effect of exogenous Bak (GFP-tagged Bak) in caspase 3-expressing cells (HeLa S3). To our surprise, HeLa S3 cells expressing GFP-Bak (as confirmed by Western blotting, Fig. 2A, lane 2) did not die. Previous studies have demonstrated that mitochondria are an important source and amplifier of apoptotic signals, and that many apoptosis-inducing factors, such as Bax, Bid and Bad, are localized in mitochondria [23]. Therefore, we next examined the mitochondrial localization of GFP-Bak by performing cotransfection and double coimmunolocalization analysis of pDs-Red1-Mito, DsRed fluorescent protein fused to a mitochondrion-targeting peptide sequence (Clontech) and GFP-Bak in HeLa S3. The two fluorescent proteins were co-localized (Fig. 2B, d) in the mitochondria. These data indicated the possibility that GFP-Bak might act via a similar mitochondrion-dependent machinery by recruiting other pro-apoptotic factors.

To examine the factors responsible for the difference in sensitivity to Bak of MCF-7 cells and HeLa S3 cells, we first examined the level of expression of the endogenous Bak in these cells (Fig. 2A). HeLa S3 (lane 2) showed a considerably higher level of expression of Bak than MCF-7 cells (lane 1). The differential effect of exogenous Bak in HeLa S3 and MCF-7 cells might be interpreted as follows. HeLa S3 cells may have escaped Bak-mediated apoptotic cell death by mechanism(s) that remain unknown in this study. These cells appear to tolerate higher levels of endogenous Bak. In contrast, MCF-7 cells have a lower level of endogenous expression and remain sensitive to Bak-mediated apoptotic cell death. We next addressed this issue by stably expressing exogenous Bak, reasoning that if the apoptotic pathway mediated by Bak is inactivated, it should be possible to isolate Bak-overexpressing stable HeLa S3 clones. However, in the presence of a functional Bak apoptotic pathway, it should be impossible to generate such HeLa S3 clones. Stable transfection and subsequent antibiotic selection of HeLa S3 cells resulted in hundreds of colonies in empty vector-transfected cells. Only a few colonies appeared from Bak-transfected cells, and when analyzed for Bak expression, these cells did not appear to have a higher level of expression than that of the control cells (data not shown). Thus, we failed to obtain HeLa S3 cells that stably overexpressed exogenous Bak. This result seems to support the conclusion that although HeLa S3 cells

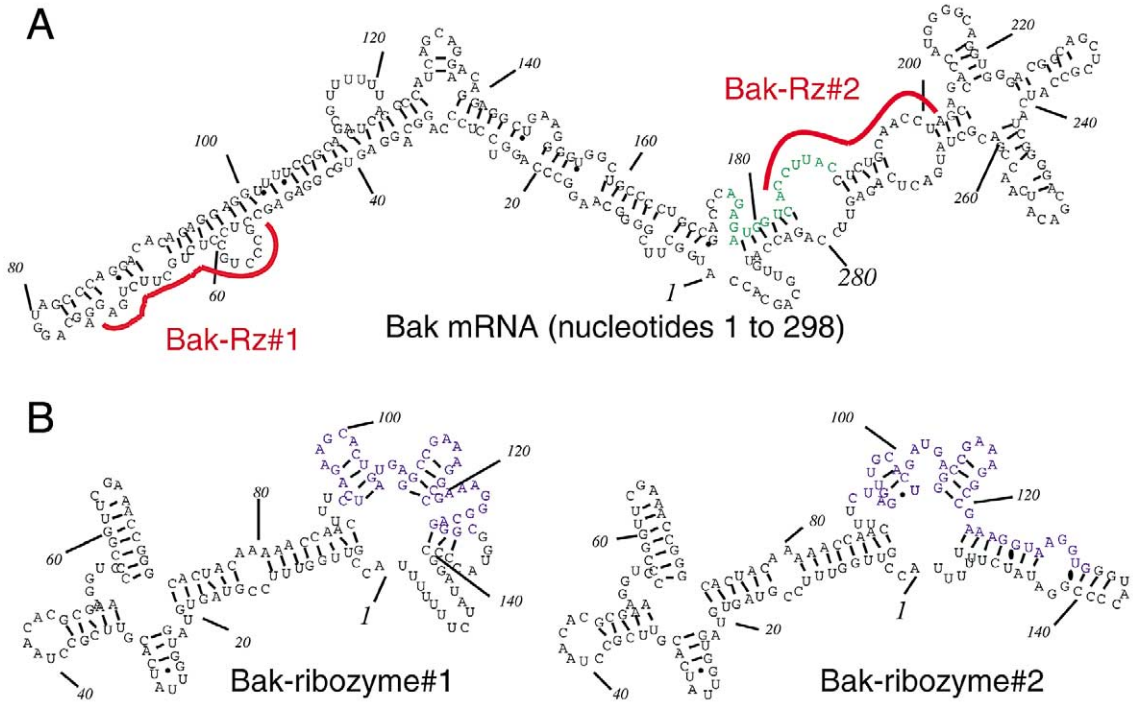

Fig. 4. The putative secondary structure of Bak mRNA and of the ribozymes used in this study. A: Partial structure of Bak mRNA corresponding to nucleotides 1–298, according to the numbering of [19]. Red lines indicate the target sites of hammerhead ribozymes, as indicated. The sequence identified by the gene discovery system based on hybrid-ribozyme libraries is indicated by green letters [11]. B: Structures of the ribozymes directed against Bak mRNA. Blue letters indicate the ribozyme.

were not as sensitive as MCF-7 cells to the effects of Bak, the apoptotic pathways induced by Bak are not inactive in these cells (see quantitative data in Fig. 3). Although HeLa S3 cells were able to escape apoptosis induced by transient overexpression of Bak, they were sensitive to the effects of constitutive Bak expression.

3.3. Construction of hammerhead ribozymes specifically targeted to Bak mRNA

To further elucidate the molecular basis of Bak action, we constructed two kinds of hammerhead ribozymes targeted specifically to Bak mRNA (Bak-ribozymes) for inhibition of the intracellular expression of Bak. Hammerhead ribozymes are catalytic RNA molecules that bind to specific RNA target molecules via complementary sequences and then cleave their RNA targets enzymatically [1–8]. The selection of target sites within Bak mRNA for cleavage by ribozymes (Fig. 4A) was based on the following four criteria: (1) the requirement for a NUX triplet at the target sequence [24–26], (2) the expectation that the target sites and their flanking regions should not form a duplex by themselves (target sites have an open structure), (3) the Bak-ribozymes would adopt the expected conformations as recognition arms of ribozymes and not form a tight duplex by themselves, and (4) the overall structure to maintain a cloverleaf motif of $tRNA^{Val}$ so that they can be transported to the cytoplasm (Fig. 4B and [4–8]). We predicted the secondary structures of Bak mRNA and the Bak-ribozymes using the Mfold program [27]. The selected target sites of the ribozymes were both CUC triplets in Bak mRNA (Fig. 4A).

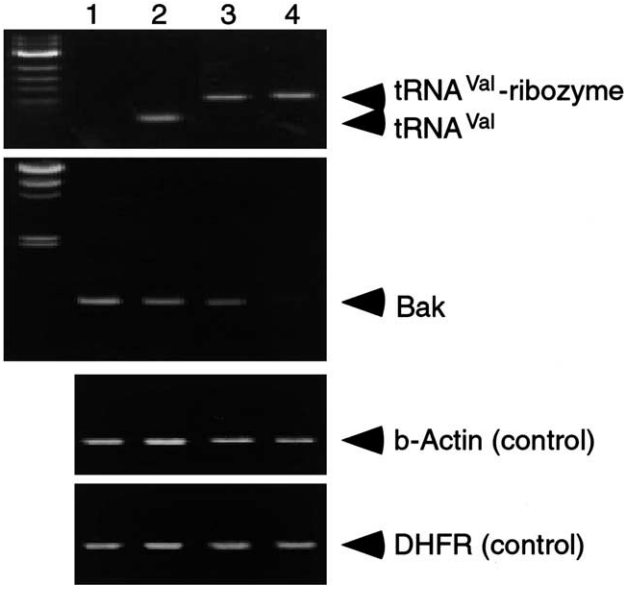

Fig. 5. Levels of expression of Bak mRNA in MCF-7 cells as determined by RT-PCR. A reduction in the level of Bak expression was detected in cells that expressed Bak-ribozyme#2 (lane 4) but not in cells that expressed Bak-ribozyme#1 (lane 3). Lane 1, non-transfected MCF-7 cells; lane 2, cells that expressed the control $tRNA^{Val}$ (ribozyme (–)); and lanes 3 and 4, cells that expressed Bak-ribozyme#1 and Bak-ribozyme#2. As internal controls for RT-PCR, results for β-actin and DHFR transcripts are also shown. The levels of expression of Bak-ribozyme#1, Bak-ribozyme#2 and the control $tRNA^{Val}$ (ribozyme (–)) were also confirmed (top panel). A reduction in level of Bak mRNA was confirmed by three independent experiments.

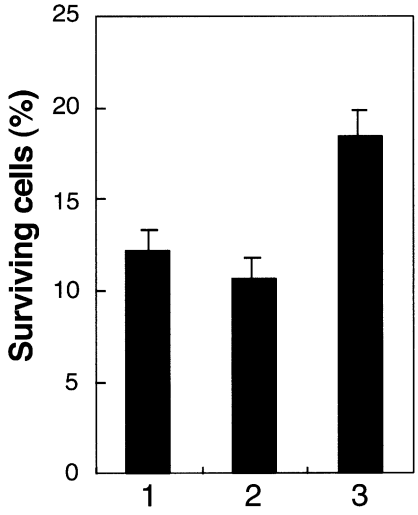

Fig. 6. Assay of TNF-α-induced cell death. The ratio of living to dead cells was determined for cells treated with TNF-α and CHX for 24 h and those that were not treated (see text for details). Results are shown for cells that expressed Bak-ribozyme#2 (column 3), non-transfected MCF-7 cells (column 1), and as a negative control, cells that had been treated with $tRNA^{Val}$ expression plasmids without a ribozyme sequence (column 2). Each column and bar represent the mean ± S.D. of results of three independent experiments performed in triplicate.

3.4. Elimination of Bak from MCF-7 cells

Plasmids that contained a Bak-ribozyme were transfected into MCF-7 cells and cells were selected by incubation in puromycin-containing medium for 4 weeks. The expression of Bak-ribozymes was confirmed by RT-PCR (Fig. 5, upper panel, lanes 3 and 4). To examine the level of Bak mRNA in Bak-ribozyme-expressing cells, we performed RT-PCR analysis for Bak. As compared to the control cells, the level of Bak mRNA was significantly reduced in MCF-7 cells that stably expressed the Bak-ribozyme#2 (Bak-Rz#2 in the middle panel in Fig. 5, lane 4). By contrast, no significant difference was found in the case of cells that expressed Bak-ribozyme#1 (lane 3). The difference between cells that expressed Bak-ribozyme#1 and Bak-ribozyme#2 might be due to the secondary conformation of Bak mRNA and its accessibility to the respective ribozymes (Fig. 4A). There were no differences in the respective levels of expression of β-actin mRNA and DHFR mRNAs, which served as internal controls, between Bak-ribozyme-expressing and control cells (Fig. 5, lower panels).

3.5. Assay of TNF-α-induced apoptosis

We subjected MCF-7 cells that stably expressed the active Bak-ribozyme#2 to an assay of TNF-α-induced apoptosis to examine the role of Bak in TNF-α-induced apoptosis. The percentage of Bak-ribozyme#2-expressing cells after exposure to TNF-α and CHX (lane 3) was somewhat greater than that of $tRNA^{Val}$-expressing (lane 2) or wild-type (lane 1) MCF-7 cells (Fig. 6). Therefore, it seems likely that Bak participates, at least to some extent, in TNF-α-mediated apoptosis in MCF-7 cells.

The cellular death process is the result of the interactions of complex pathways involving many factors and signaling molecules. Thus, a defect in Bak alone would not necessarily be expected to have a dramatic effect on apoptosis. Previously, we identified Bak using our novel gene discovery system with

randomized ribozyme libraries in the TNF-α-mediated apoptosis pathway [11]. This identification was possible because the percentage of surviving cells that expressed Bak-ribozymes after exposure to TNF-α was greater than that of wild-type MCF-7 cells, as confirmed in this study. We also confirmed by fluorescent microscopy the mitochondrial localization of Bak. This study demonstrates for the first time that in addition to its caspase-dependent function, Bak is involved in TNF-α-induced apoptosis in a caspase-independent manner.

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