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# Enhanced apoptosis by a novel gene, Bak-like, that lacks the BH3 domain

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**Abstract**

In a variety of physiological settings, cells are eliminated by apoptosis—a genetically encoded process of cellular suicide. Bak, a member of the Bcl-2 protein family, accelerates apoptosis by an unknown mechanism. We have found a novel cDNA encoding a 101-amino acid protein that possesses a Bak-like sequence in our full-length cDNA bank and termed it Bak-like. This protein shares the conserved domains BH1 and BH2 with other pro-apoptotic proteins, but lacks the BH3 domain. Database searches identified this gene on chromosome 6, which could account for the cloned *bak* and *bak-like* transcripts by alternative splicing. *Bak-like* is expressed in a wide variety of tissues. *Bak-like* is different from *bak* by Southern blots using probes with or without homology to *bak*. Despite the loss of the BH3 sequence, bak-like did enhance apoptosis, but was less potent than bak. Confocal microscopy of HeLa cells revealed that EGFP-Bak-like was located diffusely throughout the cytosol. However, upon induction of apoptosis, EGFP-Bak-like redistributed into a punctuate pattern, colocalizing with mitochondria. Like bak, the bak-like gene product directly enhanced apoptotic cell death following an appropriate stimulus.


Apoptotic cell death is centrally involved in the maintenance of tissue homeostasis in a healthy organism as well as in pathogenesis during diseased states including cancer, neurodegenerative disorders, autoimmune diseases, and viral infection [1]. Although the precise mechanisms by which apoptosis is regulated remain unknown, it is clear that in various species, programmed cell death is controlled by an evolutionarily conserved cellular machinery incorporating sets of genes which include ced-3/caspase, ced-4/Apaf-1, and ced-9/Bcl-2 [1–3].

The Bcl-2 family of proteins plays a central regulatory role in apoptosis. Anti-apoptotic (Bcl-2, Bcl-xL and Bcl-w, Mcl-1, and A1/Bfl-1) and pro-apoptotic (Bax, Bak, Bok, Bik, Bad, Bid, Bim/BOD, and Hrk) members of this family have been characterized [4–9]. These proteins can form both homo- and heterodimers and, as a consequence, can function either independently or in concert to regulate apoptosis [10]. Dimerization of Bcl-2 family members involves interactions between conserved amino acid sequences known as Bcl-2 homology (BH) domains. Four of these domains (BH1, BH2, BH3, and BH4) have now been identified and they appear to play a crucial role in specifying the pro- or anti-apoptotic properties of a given family member [11–14].

Anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, possess all four BH domains and both BH1 and BH2 appear necessary for their dimerization with Bax and for the suppression of apoptosis [15–17]. In contrast, the pro-apoptotic proteins Bax, Bak, and Bok lack a recognizable BH4 domain while Bid, Bad, Bik/Nbk, Hrk, Bim, Blk, and EGL-1 are characterized by the presence of only a BH3 domain ("BH3 only" proteins). Outside of this region these proteins display considerable sequence diversity.

The binding of Bad to Bcl-xL through its BH3 domain is required to promote apoptosis [18,19]. However,

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it has now been shown that the pro-apoptotic activity triggered by this domain does not always depend on its interaction with anti-apoptotic proteins. Thus, Bid BH3 mutants that lack the ability to bind Bcl-2, but that retain the ability to bind Bax, are still potent activators of apoptosis [20]. These observations suggest that the mechanisms by which BH3 domains trigger apoptosis may vary from one family member to another and that this may reflect their involvement in multiple pathways leading to cell death.

Bak has been independently identified in three different laboratories. Farrow et al. [21] reported the identification of three cellular proteins that bind E1B 19K [21]. One of these is a new member of the Bcl-2 family, which they have called Bak (for Bcl-2 homologous antagonist/killer). Chittenden et al. [5] and Kiefer et al. [6] simultaneously isolated 'Bak' among the Bcl-2 homologue clones using PCR with the primers specified to BH1 and BH2 domains. Bak has domains which bind to Bcl-2, Bcl-xL, and E1B 19K. Within Bak, only the BH3 domain has binding activity for Bcl-2 and Bcl-xL, and this domain also enhances apoptosis [16]. Moreover, enforced expression of Bak induces rapid and extensive apoptosis of many cell types [21]. In this study, we identify a Bak homologue, termed Bak-like, from a human full-length cDNA bank. Additionally, we explore the implication of Bak-like in apoptosis.

## Materials and methods

*DNA sequencing*. A plasmid containing *bak-like* DNA was reacted in a primer cycle sequence kit (Perkin–Elmer, USA). The nucleotide sequence of the reacted product was determined with a DNA sequencer (Perkin–Elmer Biosystems, model 377).

*Northern blot analysis*. The *bak-like* gene was labeled with $^{32}$P using a random primer labeling kit (Takara, Japan) and used as a probe for a 267-bp nucleotide sequence—corresponding to 83–349 of *bak-like*. A Multiple Tissue Northern Blot Filter (Clontech, USA) was hybridized with this probe at 50 °C in QuickHyb hybridization solution (Clontech, USA). The membrane was washed in a solution containing 0.1× SSC and 0.05% SDS at 50 °C. It was then exposed to X-ray film at 80 °C for 12 h.

*Polymerase chain reaction*. mRNA was extracted from colon, ovary, peripheral blood leukocytes, prostate, small intestine, spleen, testis, thymus, breast carcinoma (GI-101), lung carcinoma (CX-1), colon adenocarcinoma (CX-1), lung carcinoma (GI-117), prostatic adenocarcinoma (GI-102), and pancreatic adenocarcinoma (GI-103) tissues. These mRNA species were reversely transcribed into cDNAs (Clontech, USA). Two combinations of polymerase chain reaction (PCR) primers for *bak-like* were used: sense 5′-TTCGAG GACTTTTCT-3′ and anti-sense 5′-GAGGGATTGCACAG-3′ for region of *bak-like*; sense 5′-GAGTATCCAAGGACT-3′ and anti-sense 5′-GGAGAAACAAGGTGG-3′ for the region of *bak* non-homologue. The primer for *bak* was designed to correspond to the sequence of the open reading frame (ORF) region. PCR was conducted as follows: predenaturation for 3 min at 94 °C, denaturation for 45 s at 94 °C, annealing for 30 s at 55 °C, and elongation for 90 s at 72 °C for a total of 30 cycles.

*Southern blot analysis*. Genomic DNA (10 μg) of whole blood (Promega, USA) was digested with *Sac*I, *Hin*dIII, *Xba*I, and *Eco*RI, and then separated by electrophoresis on an 0.8% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham–Pharmacia Biotech, Japan). This was followed by hybridization at 60 °C in Quick hyb Buffer (Clontech, USA) with a $^{32}$P-labeled 210-bp nucleotide sequence that corresponded to 327–536 of *bak* and a 267-bp nucleotide sequence—corresponding to 83–349 of *bak-like*. After hybridization, the membranes were washed in 0.1× SSC, 0.05% SDS at 50 °C before exposure to X-ray film for 24 h at −80 °C.

*Cell survival rate*. HeLa cells ($2 \times 10^5$ cells/well) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. One day later, cells were transfected using Gene PORTERTM (Gene Therapy Systems, USA) with the enhanced green fluorescent protein (EGFP) expression vector with or without EGFP fusion vector containing *bak* or *bak-like*, together with 1/10 to 1/20 amounts of an indicator plasmid pCMV-β-gal to facilitate the identification of transfected cells. Inclusion of a 10-fold excess of expression vectors, as compared with the pCMV-gal reporter plasmid, ensured that most of the β-galactosidase-expressing cells also expressed the proteins under investigation. Cells were incubated with plasmids in a serum-free medium for 4 h, followed by the addition of fetal bovine serum to a final concentration of 5% and further incubation for 5 h. After an additional culture in fresh medium for 24 h, cells were fixed using 0.25% glutaraldehyde and stained with X-gal to detect β-galactosidase expression (Promega, USA). Data are expressed as the percentage (mean ± SEM) of viable cells as compared with the control group [26].

*Confocal microscopy*. Cells used for confocal microscopy were transfected as described above on 1.5-μm thickness glass coverslips that had been pretreated with 5 μg/ml poly-L-lysine. After 24–48 h, cells were prepared for microscopy by incubation with 20 ng/ml of a mitochondrion-specific dye (Mitotracker Red CMXRos; Molecular Probes, Eugene, OR) for 30 min. Cells were transferred to a sealed mounting chamber containing fresh medium which was supplemented with 1–3 μM staurosporine (STS). Images were collected on a microscope (model LSM 410 with a 40 × 1.2NA Apochromat objective; Carl Zeiss, Thornwood, NY). The 488- and 568-nm lines of a krypton/argon laser were used for fluorescence excitation of EGFP and Mitotracker Red CMXRos, respectively. The temperature of the specimen was maintained at 37 °C with an airstream incubator.

## Results and discussion

### *Lack of a BH3 domain in the sequence of bak-like*

Kato et al. [22] previously reported the construction of the bank composed of full-length cDNAs encoding human proteins. In this cDNA bank, we found a *bak* homologue clone and named it '*bak-like*.' *Bak-like* had 2014 mRNA bases coding for a protein of 101 amino acids. The 350–536 bp region of *bak-like* was homologous to *bak* (Fig. 1). BH1(12–22 amino acids), BH2 (52–67 amino acids), and TM (91–101 amino acids) domains were identified (Fig. 1, underlined). Bak-like exhibits marked structural similarity to Bak, particularly in the BH1 and BH2 domains. However, unlike bak, BH3 domain was not present in Bak-like (Fig. 2). Among its amino acid sequence, the Bak-like N-terminal, consisting of 10 amino acids, and the C-terminal with 33 amino acids were not homologous to Bak. From a homology search in human genome databases, we identified a putative gene underlying this transcript on chromosome 6 (GenBank Accession Nos. Z93017.6|HS291J10).

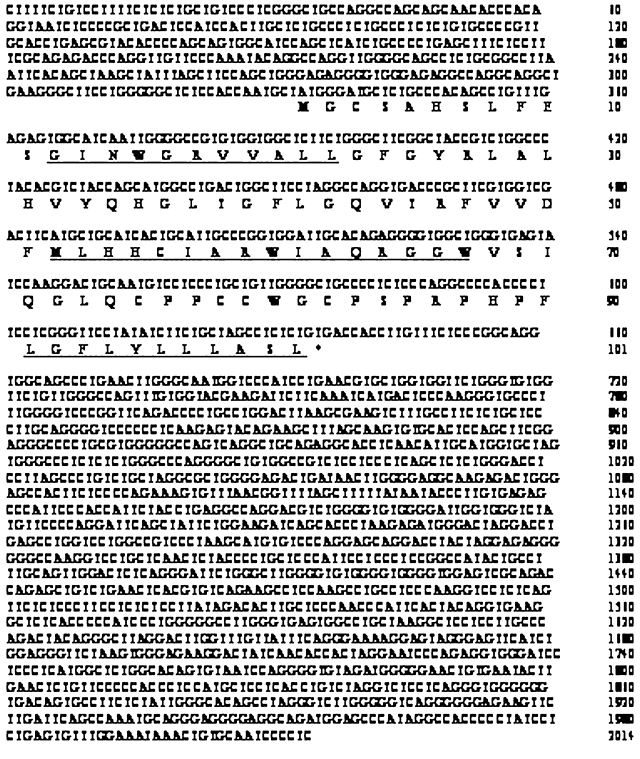

Fig. 1. Primary structure of *bak-like*. *Bak-like* has 2014 mRNA bases coding for 101 amino acids. The 350–536 bp region of *bak-like* is a *bak* homologue. BH1 (12–22 amino acids), BH2 (52–67 amino acids), and TM (91–101 amino acids) domains were identified (underlined).

BH1 and BH2 domains have been known to form dimers with Bcl-2 family proteins. Recent studies using crystallography, computer modeling, and membrane potential recording have established that the amphipathic BH3 domain in some pro-apoptotic Bcl-2 proteins might regulate apoptosis by binding to a hydrophobic cleft formed by the conserved BH1, 2, and denced by Bcl-2 and Bcl-xL [23,24]. Interestingly, Bak-like did not contain a BH3 domain that might be necessary to induce apoptosis, although it is a novel splice variant of the *bak* gene. Since pro-apoptotic activity triggered by the BH3 domain is not always dependent on its interaction with anti-apoptotic proteins, we further investigated the role of the bak-like gene in apoptosis.

### *Expression of bak-like mRNA in normal and cancer cells*

To elucidate the expression pattern of *bak-like*, mRNA extracted from cancerous and normal tissues was analyzed by Northern blotting. The *bak-like* mRNA had a very similar length (2.4 kb) to *bak* mRNA and was expressed in most cancerous and normal tissues (Fig. 3).

To exclude the possibility that *bak-like* is formed artificially during the cDNA library processing, we performed PCR to cDNA library templates prepared from mRNA of cancerous and normal tissues. This was performed with primers manufactured to nucleotide sequence 536–638, which has no homology to *bak*, and nucleotide sequence 201–836 of full-length *bak*. The *bak-like* gene was expressed in both cancerous and normal cells and tissues, but its pattern of expression was different to that of *bak* (Fig. 4). From our results, we conclude that *bak-like* is an endogenous molecule rather than an artificial one.

### *Bak-like is an alternative splice variant of bak*

To investigate whether *bak-like* might arise from alternative splicing of *bak* mRNA, Southern blot analysis

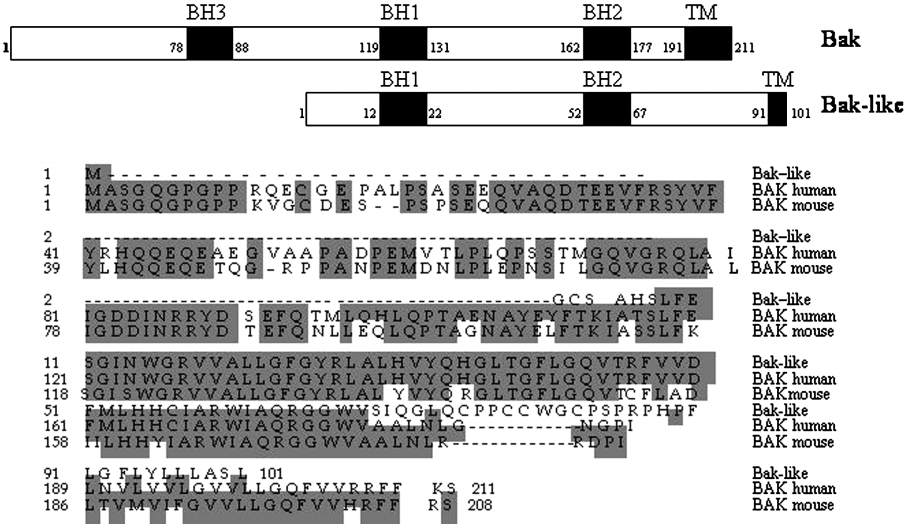


Fig. 2. Amino acid sequence comparisons Bak-like with human and mouse Bak (for Bcl-2 homologous antagonist/killer). Bak-like exhibits marked structural similarity to Bak, particularly in the BH1 and BH2 domains. BH3 domain was not present in Bak-like, unlikely Bak. Among its amino acid sequence, the Bak-like N-terminal, consisting of 10 amino acids, and the C-terminal with 33 amino acids were not homologous to Bak.

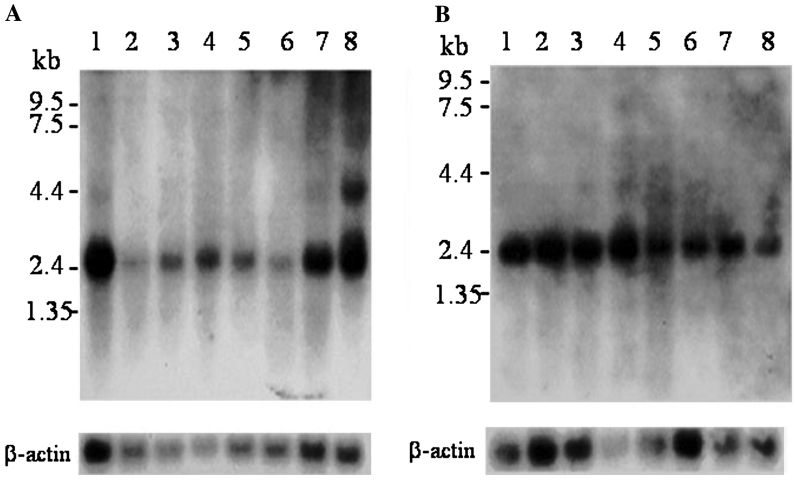


Fig. 3. Northern blot analysis of *bak-like* expression. (A) Multiple tissue Northern blot (human cell line). Lanes: 1, promyelocytic leukemia HL-60; 2, HeLa cell S3; 3, chronic myelogenous leukemia K562; 4, lymphoblastic leukemia MOLT-4; 5, Burkitt's Lymphoma Raji; 6, colorectal adenocarcinoma SW480; 7, lung carcinoma A549; and 8, melanoma G361. (B) Multiple tissue Northern blot (human tissues). Lanes: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; and 8, peripheral blood leukocytes.

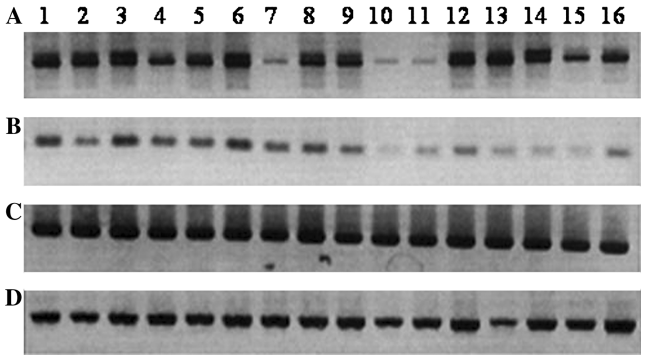


Fig. 4. Expression of *bak-like* and *bak* on a transcriptional level. Template cDNA libraries for PCR derived from: 1, colon; 2, ovary; 3, peripheral blood leukocytes; 4, prostate; 5, small intestine; 6, spleen; 7, testis; 8, thymus; 9, breast carcinoma (GI-101); 10, lung carcinoma (LX-1); 11, colon adenocarcinoma (CX-1); 12, lung carcinoma (GI-117); 13, prostatic adenocarcinoma (PC3); 14, colon adenocarcinoma (GI-112); 15, ovarian carcinoma (GI-102); and 16, pancreatic adenocarcinoma (GI-103). (A) *bak-like* (nucleotides 1–2014). (B) *bak-like* (nucleotides 536–638). (C) *bak* (nucleotides 201–836). (D) G3PDH.

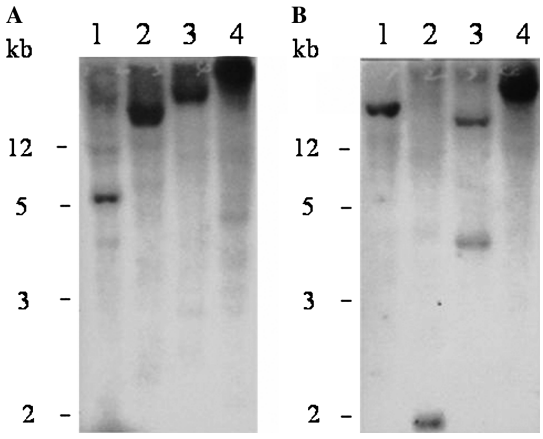


Fig. 5. Southern blot analysis. Human genomic DNA 10 μg was digested with the indicated restriction enzymes (lane 1, *Sac*I; lane 2, *Hin*dIII; lane 3, *Xba*I; and lane 4; *Eco*RI) and probed with a 210-bp cDNA fragment (*bak* nucleotides 327–536) (A) or a 267-bp cDNA fragment (*bak-like* nucleotides 83–349) (B).

was executed. Genomic DNA was treated with restriction enzymes prior to Southern blot analysis. We used two different probes, one directed to nucleotides 327–536, which bears homology to *bak* (Fig. 5A), and the other corresponding to nucleotides 83–349 which does not bear homology to *bak* (Fig. 5B). This indicated that these two probes identified distinct expression patterns, suggesting that *bak-like* might be an alternatively spliced variant of *bak*.

### *Bak-like enhances apoptosis in transfected cells*

Although Bak-like has no BH3 domain, which is known to promote apoptosis, the fact that Bak-like is homologous to Bak and that it is an alternative splicing variant of Bak suggests that Bak-like may also be pro-apoptotic. Thus, we investigated the ability of Bak-like to regulate apoptosis. Despite the loss of the BH3 sequence, bak-like did enhance apoptosis, but was less potent than bak (Fig. 6).

We also investigated the ability of Bak and Bak-like to dimerize using yeast two-hybrid methodology. Although Bak-like contains BH1 and BH2 domains, it failed to dimerize with Bak (data not shown).

### *Translocation of Bak-like from the cytosol to mitochondria during apoptosis*

We examined the location of Bak-like in individual normal cells using EGFP fusion constructs as these have

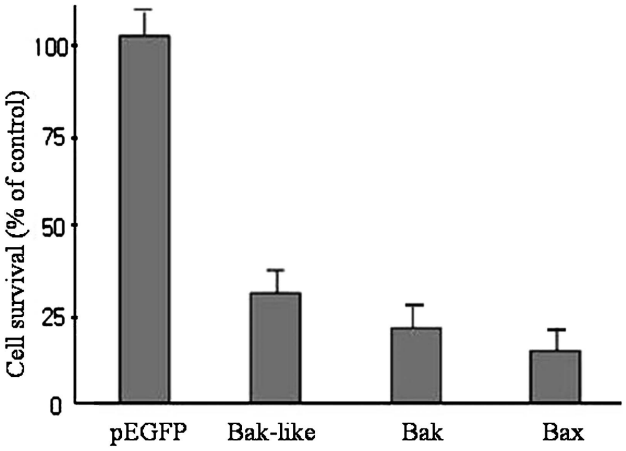


Fig. 6. Comparison between Bak, Bak-like, pEGFP, and Bax in inducing apoptosis. Bak-like enhances apoptosis but does so less potently than Bak.

previously been successfully used to study the subcellular localization of various proteins [25–27]. The localization of transiently expressed EGFP-linked fusion proteins was visualized in live cells using confocal microscopy. In HeLa cells, EGFP alone has a diffuse distribution, filling the entire cell as reported previously [28]. EGFP-Bak-like was also distributed diffusely throughout the cytosol in HeLa cells. However, upon induction of apoptosis with STS, EGFP-Bak-like redistributed into a punctuate pattern, colocalizing with mitochondria (Fig. 7). STS-treated cells underwent cell shrinkage and blebbing of the cell membrane, a morphology indicative of apoptosis. EGFP-Bak-like markedly changed its intracellular distribution, translocating within cells during apoptosis from a diffuse to a punctuate pattern. This change was not observed with EGFP alone. Mitochondrial staining of EGFP-Bak-like-expressing HeLa cells undergoing apoptosis suggests that the majority of EGFP-Bak-like attaches to, or enters, mitochondria.

From these results, we concluded that Bak-like could promote apoptosis. Although Bak-like contains BH1 and BH2 domains, it did not dimerize with Bak. Furthermore, Bak-like enhanced apoptosis, although it does not contain a BH3 domain, which was previously assumed to be necessary for promoting apoptosis. An intact bak BH3 domain, which was previously believed to be important for dimerization with selective anti-apoptotic Bcl-2 proteins and for cell death, appears not to be essential for apoptosis regulation. Similar to Bad, the mechanism of action of Bak-like might be different from that of Bak or other Bcl-2 family proteins. It is possible that Bak-like acts directly on the mitochondrial membrane, since it retains a C-terminal hydrophobic transmembrane (TM) region as a membrane anchor. Therefore, further studies on Bak-like, particularly with respect to binding proteins, are warranted.

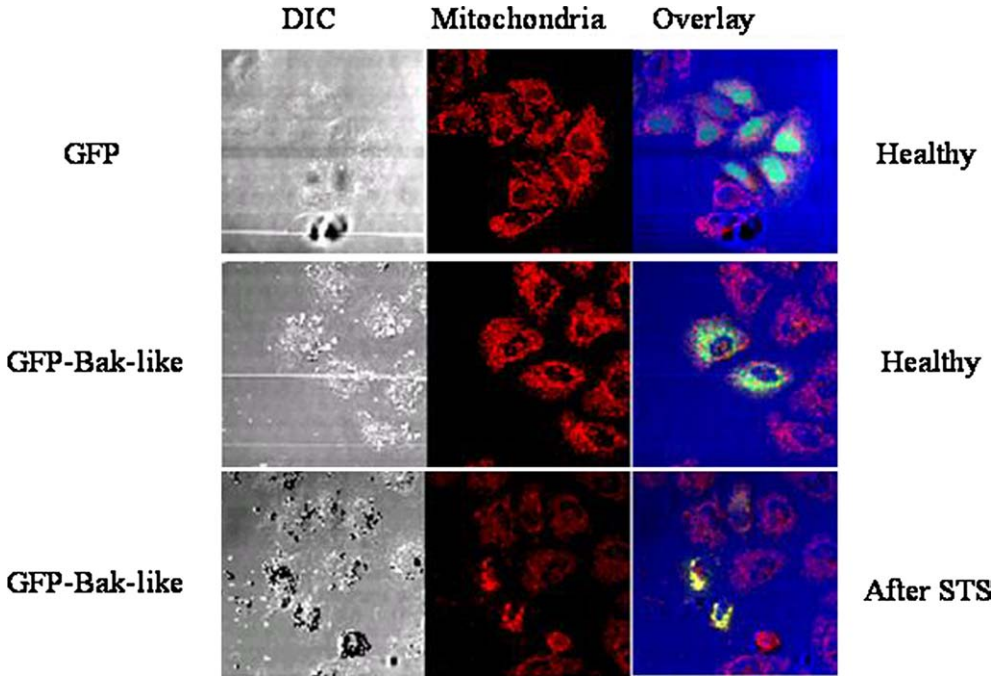


Fig. 7. Distribution of GFP-fusion proteins expressed in live HeLa cells, before and after STS treatment. At 24–48 h after transfection, cells were examined by confocal microscopy. Each field was visualized by laser fluorescence to detect GFP and by DIC to illustrate cell morphology. Native GFP distributes throughout healthy cells. EGFP-Bak-like displays a diffuse distribution throughout the cytosol in HeLa cells. However, upon induction of apoptosis with STS, EGFP-Bak-like redistributes to form a punctuate pattern colocalizing with mitochondria. STS-treated cells undergo cell shrinkage and blebbing of the cell membrane, a morphology indicative of apoptosis. EGFP-Bak-like undergoes a change in its intracellular distribution, relocating within cells during apoptosis from a diffuse to a punctuate pattern.

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