Correspondence

Evolutionarily conserved Bok proteins in the Bcl-2 family

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Apoptosis is under tight control of rather complex regulatory circuits in which proteins of the Bcl-2 family act as a critical life–death decision point. The Bcl-2 family consists of a growing number of proteins [1]. In *Caenorhabditis elegans* there is only one member, *ced-9*, existing. In mammalian cells the regulation of apoptotic process by the Bcl-2 family appears to be very complex. More than 10 members of the Bcl-2-related proteins have been identified so far.

Previously, we cloned a human Bok gene (Hbok) from a human infant brain library (GenBank accession number AF174487). By search of the expressed sequence tags (EST) database/*Drosophila* genome sequence database using the amino acid sequences of HBcl-xL and HBok, and by use of polymerase chain reaction (PCR), we further identified two *Drosophila* and a chicken Bok homologue (Fig. 1). Briefly, a Blast Search of the GenBank database ESTs was performed and a partial nucleotide sequence of a *Drosophila* cDNA was identified. Subsequently, 5′-RACE PCR was performed on mRNA of *Drosophila* Schneider L2 (S2) cells to amplify the 5′ sequences, which were then to be compared to genomic sequences from Flybase. This resulted in obtaining the entire coding region containing a 978 nt open reading frame (Fig. 1A). The predicted amino acid sequence showed 35% identity to the human Bok protein, but about 25% identity to other Bcl-2-related proteins. This gene was then named Dbok. Dbok is identical to Drob-1 recently published by Igaki et al. [2], but longer than Debcl from Colussi et al. [3]. Alignment of cDNA sequence along with genomic sequences from the *Drosophila* genome sequence database revealed that the genomic sequence of Dbok contains three exons. This result and a further search of the *Drosophila* genomic sequence database recovered a sequence which represents another distant Bcl-2-related gene, named Dbx (Fig. 1A).

In addition, we identified a chicken Bok from three chicken cDNA clones, which contain partial nucleotide sequences. Sequence analysis resulted in identification of a 1158 nt full-length cDNA which contains a 641 nt coding sequence. Its amino acid sequence shares 81% identity with that of human Bok (Fig. 1A).

The analysis of Bok proteins with other members in the Bcl-2 family revealed that they contain all four different BH domains, BH1, BH2, BH3 and BH4 (Fig. 1A). But both *Drosophila* homologues contain two BH4 domains, whereas chicken and human Bok contain only one BH4 domain (Fig. 1B). The alignment shows that a highly conserved stretch of amino acids, ITWGK, is present in the BH1 domain. It differs in this region from other Bcl-2 family members and is a characteristic for this sub-group in the Bcl-2 family (Fig. 1A).

Dbok has a long N-terminus with an additional BH4 domain as compared to other Bcl-2 related proteins. The topology prediction of the membrane regions of Dbok showed that addition to a candidate membrane-spanning segment of amino acids 221–241 (BH1 domain) and a putative hydrophobic membrane anchor between amino acid 290 and 310, both of which have been shown to have important features, the N-terminal sequence also contains a transmembrane segment within amino acids 14–34 [4]. This region is not present in vertebrates. To explore the structure and function relationship of Dbok, we have used an apoptosis assay, in which apoptotic REF52 cells round up at an early apoptotic stage and detach from the surface of the dish [5]. The DNA encoding full-length Dbok was cloned into the pcDNA3 expression vector. The constructs were transfected into REF52 rat fibroblasts together with a vector (pEGFP-N1) encoding the green fluorescent protein (GFP). GFP served as marker for successfully transfected cells. 15 h after transfection, apoptotic REF52 cells were counted under a fluorescence microscope [5]. As shown in Fig. 1C, 36% of cells transfected with a plasmid carrying the full-length Dbok cDNA became apoptotic, whereas the expression of Bax induced 38% of apoptosis. Furthermore most of the Dbok-transfected cells could be rescued by co-expression of Bcl-xL or BFL-1. The N-terminal-truncated Dbok construct, Dbok-N, in which the first BH4 domain has been deleted, did not lose the apoptotic activity in REF52 cells, indicating that the N-terminal region is not necessary for its apoptotic function. One possible role of this region is not directly involved in the cell killing but rather in mediating the conformation of the protein through apoptosis stimuli. However, the C-terminal-truncated construct, Dbok-C, which lacks a putative transmembrane domain and the Dbok-BH3, which is a BH3 domain deletion construct, lost their killing activity. These results are consistent with the previous observations that mutants of the Bcl-2-related proteins lacking the transmembrane domain in the C-terminus are functionally ineffective. The loss of function of the C-terminal mutant indicated that the localization of Dbok is important for its function. Thus, Dbok is a pro-apoptotic protein which functions through the BH3 domain. The C-terminal sequence and the BH3 domain of Dbok are indispensable for the pro-apoptotic function but the N-terminal sequence is not required for its apoptotic potential.

At this stage we do not know the function of Dbx yet. The amino acid sequence of Dbx is very similar to that of Dbok and Dbx also contains the BH4 and BH3 domains. Most interestingly a proline residue is present in the middle of the BH3 domain, which may well change conformationally the local helical structure of the BH3 domain based on the structural analysis of the Bcl-xL and Bfl-1 [5]. Further experiments are necessary to determine the function of this protein. Like Bok proteins from *Drosophila*, rat and human, chicken Bok may well function as a pro-apoptotic protein.

Most recently, two Bcl-2 homology proteins have been isolated from two different species of sponges [6]. Here we report the identification of three Bok protein genes from *Drosophila*, human and chicken. These results indicate that the apoptosis pathway is evolutionarily conserved even from sponges to human and the complexity of regulation of apoptosis fits to the evolutionary relationships among animals.

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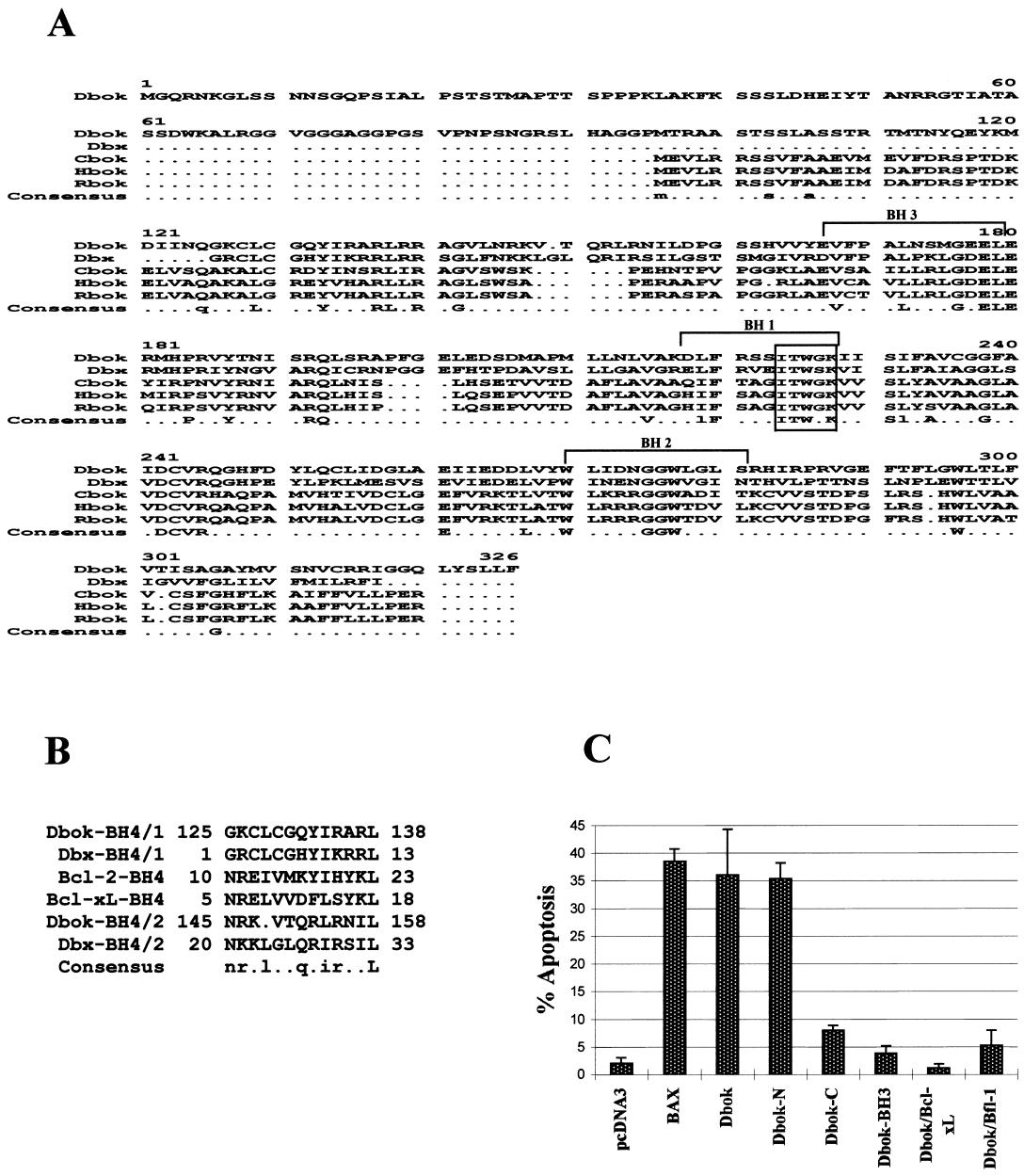

Fig. 1. Isolation of cDNAs encoding Bok proteins from *Drosophila* and chicken. A: Alignment of Bok proteins from *Drosophila* (Dbok), chicken (Cbok), rat (Rbok) and human (Hbok). EMBL/GenBank/DDBJ accession numbers for human and chicken Bok are AF174487 and AF275944, respectively. The putative BH1–3 domains of Bok are marked. The second putative BH4 domains are only present in Dbok and Dbx. The stretch of ITWGK is shown in the box. A 622 nt EST sequence (number AI513093) was found from *Drosophila* ESTs, named DBok. 5′ RACE-PCR (Roche Molecular Biochemicals) was performed to clone further 5′ sequences from total RNAs of S2 cells using two primers (5′-CAGCGTCAACCATCCCAAGAA-3′) and (5′-CGAATTCCTAGAACAGCAGCGAATA-3′). Comparison of the sequence of PCR products to the Genome database of the *Drosophila* genome project from Flybase revealed that three BAC sequences (accession numbers AC007593, AC018035 and AC007624) matched 100% of the DBok sequence. The others with accession numbers AC016794, AC020266 and AC007473 were highly homolog to Dbok but represent another gene, which was named Dbx. Finally, Dbok cDNA was cloned from the total RNAs of S2 by RobusT RT-PCR (Finnzymes) using the primers (5′-CAAGCTTCGATGATGGATATCATCAACCAGG-3′) and (5′-CGAATTCCTAGAACAGCAGCGAATA-3′). Search of the EST GenBank database with the human Bok amino acid sequence revealed three chicken cDNA clones (cDNA clone 12d8r1, 14il8r1 and 3f12r1 with accession numbers AJ393173, AJ392631 and AJ397849), each of which contains partial sequences encoding chicken Bok. B: Alignment of the putative BH4 domains of Dbok and Dbx with the BH4 domains of Bcl-2 and Bcl-xL. C: Dbok function as a pro-apoptotic protein. REF52 cells were transfected using either Lipofect AMINE Reagent (Gibco BRL) with 1 μg of plasmids encoding GFP (pEGFP-N1, Clontech) together with 1 μg of pcDNA3, carrying the genes to be tested. Human Bcl-xL, Bcl-2, Bax, BFL-1 and the DBok were sub-cloned into pcDNA3 (Invitrogen) [5]. Forward and reverse primers used to construct the deletion mutants are listed as following: Dbok-N: (5′-CAAGCTTCGATGGCAGGAGTCCTCAACCGGA-3′) and (5′-CGAATTCCTAGAACAG-CAGCGAATA-3′), Dbok-C:(5′-CAAGCTTCGATGATGGATATCATCAACCAGG-3), (5′-CGAATTCCTAGAACGTAAATTCGCCGA-3′). Dbok-BH3 is a 7 residue in-frame deletion mutant lacking ALNSMGEE of the BH3 domain. The mutant was constructed using a primer (5′-GGTCTATGAAGTTTTCGAACTGGAGAGGATGCA-3′) by the 'splicing' PCR method. The pcDNA3 plasmid served as control. 15 h after the transfection, the amount of apoptotic cells were counted by microscopic examination. At least 100 cells from each individual culture were analyzed. Apoptosis of cells is expressed (in %) as apoptotic cells per total cells, with the standard deviation of the assay in three independent experiments.

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