



Short communication

BCL-G as a new candidate gene for immune responses in pigs: Bioinformatic analysis and functional characterization

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ABSTRACT

BCL-G, also known as Bcl2-like14, is a unique member of the Bcl-2 family that plays an important role in regulating apoptosis in humans. In the present study, we assessed the biological activities of porcine BCL-G (pBCL-G). The open reading frame (ORF) of pBCL-G covered 990bp and encoded 329 amino acids. The genomic structure of the pBCL-G gene was also determined. The deduced amino acid sequence of the pBCL-G cDNA was highly identical to homologs in other species. Furthermore, domain prediction showed that pBCL-G protein contains BH2 and BH3 domains, which are typical domains of the Bcl-2 family. Phylogenetic analysis indicated that BCL-G may function differently among species. Subcellular localization analysis showed that GFP-pBCL-G fusion protein is distributed in the nucleus and cytoplasm. Flow cytometric analysis proved that pBCL-G is a pro-apoptotic factor. This study is useful for understanding pBCL-G and offers a potential molecular model for the investigation of diseases related to human BCL-G.

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1. Introduction

Apoptosis is an ordered and orchestrated cellular process that occurs normally during development and aging and acts as a homeostatic mechanism to maintain cell populations in adult tissues. The process is also a defense mechanism in immune reactions or when cells are damaged by disease or noxious agents (Carol Mattson Porth, 2010; Mohan, 2010). Many pathways by which induction of apoptosis occurs are known but only two, the intrinsic and extrinsic pathways, have been demonstrated in detail (Reed, 2000). Bcl-2 family proteins, which operate immediately upstream of mitochondria, regulate the intrinsic

pathway. Bcl-2 family proteins consist of pro-apoptotic and anti-apoptotic members. Pro-apoptotic Bcl-2 family proteins have the Bcl-2 homology 3 (BH3) domain (Bcl-2 family proteins have four conserved Bcl-2 homology domains named BH1 to BH4) in common, an interacting domain that is both necessary and sufficient for the killing action (Reed, 2000). The BCL-G gene is a member of the pro-apoptotic Bcl-2 family of proteins originally identified in humans (Guo et al., 2001). BCL-G has been implicated in prostate cancer and systemic lupus erythematosus and could interact with BCL-X_L, MNSFβ, p53, TEF, DBP, JAB1, and MELK (Benito et al., 2006; Guo et al., 2001; Lin et al., 2007; Liu et al., 2008; Miled et al., 2005; Nakamura and Tanigawa, 2003).

In this study, we characterized the features of the porcine BCL-G (pBCL-G) gene and investigated its subcellular localization and function. Pigs serve as a suitable model for studying intestinal development or disease because they have a close phylogenetic relationship with humans, share many physiological similarities with humans, and

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offer several breeding and handling advantages (Vodicka et al., 2005). Our results contribute useful information on the pBCL-G gene and offer a potential molecular model for studying diseases related to human BCL-G.

2. Materials and methods

2.1. Cells and pBCL-G gene

Porcine kidney-15 (PK15) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Hyclone), supplemented with 10% fetal bovine serum (FBS) (Hyclone) under humidified air containing 5% CO₂ at 37 °C. pBCL-G cDNA was cloned from porcine spleen by reverse transcription-PCR (RT-PCR) (Jiang et al., 2010) and stored in the Laboratory of Virology, Immunology and Bioinformatics, College of Veterinary Medicine, Northwest A & F University.

2.2. Bioinformatic analysis of sequence and structure

Initially, the cDNA and corresponding genomic sequences were analyzed using Spidey software (<http://www.ncbi.nlm.nih.gov/spidey>) to establish the exon-intron organization. Then, the mRNA sequence was entered into Open Reading Frame (ORF) Finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) to determine its ORF and acquire amino acid sequences. The amino acid sequence was analyzed using Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) software. The theoretical isoelectric point (pI) and molecular weight (Mw) of pBCL-G protein were computed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Domains were predicted using the SBASE web tool (<http://hydra.icgeb.trieste.it/servers/protein/sbase>) and SWISS-MODEL Workspace (Arnold et al., 2006).

2.3. Phylogenetic and molecular evolutionary analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA4 software (Tamura et al., 2007). The sequences compared were deposited in GenBank (GenBank Nos. XP_002924875, NP_001015544, XP_534887, XP_002752184, XP_001501335, NP_620048, XP_001083791, AAH25541, XP_003265555, XP_002712712, XP_002822986, XP_001152279, NP_001019509, and JN628029). The evolutionary history of the gene was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkanndl and

Pauling, 1965) and are presented in units of number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). A total of 204 positions were included in the final dataset.

2.4. Plasmid construction and transfection

cDNAs containing the ORF of pBCL-G without additional flanking sequences were generated by PCR with the following primer pair: forward, 5'-CAAAGCTTATATGTGCACCACCAGCACC-3' and reverse, 5'-CCGATCCTCAGTCTACTTCTTCATGGG-3'. The resulting PCR products were digested with restriction endonucleases and subcloned into the *Bam*HI and *Hind*III sites of pEGFP-C1. The recombinant plasmid was named pEGFP-pBCL-G. PK-15 cells were cultured first in DMEM containing 10% FBS in 6-well plates under humidified air containing 5% CO₂ at 37 °C for 24 h and then in medium without serum for 1 h. Cells were transiently transfected with 4 μg of the pEGFP-pBCL-G plasmids combined with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The expression of GFP-pBCL-G was analyzed by inverted fluorescent microscopy (Nikon).

2.5. Intracellular distribution of porcine BCL-G protein

About 24 h after transfection, cells were washed with PBS and then incubated at 37 °C for 5 min with 10 μM Hoechst 33342. The samples were analyzed by an Olympus Fluoview™ FV1000 confocal microscope (Olympus). The results were processed using FV1000 Viewer software.

2.6. Analysis of apoptosis by flow cytometry

We detected the apoptosis of PK-15 cells transiently expressing GFP-BCL-G induced by poly I:C (an interferon inducer and an artificial mimic of viral RNA that induces immune responses similar to a viral infection) (Huang et al., 2010) to study whether or not pBCL-G has any function related to innate immune responses. The flow cytometric system (CyFlow Cube6, Partec) and annexin V-PE/7-AAD-kit (BD Pharmingen) were used according to the manufacturers' instructions. PK-15 cells expressing GFP-pBCL-G or GFP were plated in 6-well plates at a concentration of 2.5×10^5 cells per well (2 mL/well) and cultured in DMEM supplemented with 10% FBS under humidified air containing 5% CO₂ at 37 °C. When about 80% confluence was observed, the medium was replaced with fresh challenge medium. Then, poly I:C (Sigma-Aldrich) was added at 4 μg/mL. Cells were harvested 18 h after treatment. After washing with D-Hanks, the cells were detached with trypsin and 5×10^5 cells of each group were resuspended in binding buffer and incubated with annexinV-PE/7-AAD for 15 min at 25 °C. Dead cells and debris were excluded by selective gating based on electronic cell volume. About 20,000 events were collected from each sample. Using dual-parameter staining and CyView8.5 software (Partec), early and late apoptosis were quantified. In preliminary experiments, unstained medium-treated cells were used to define living cells

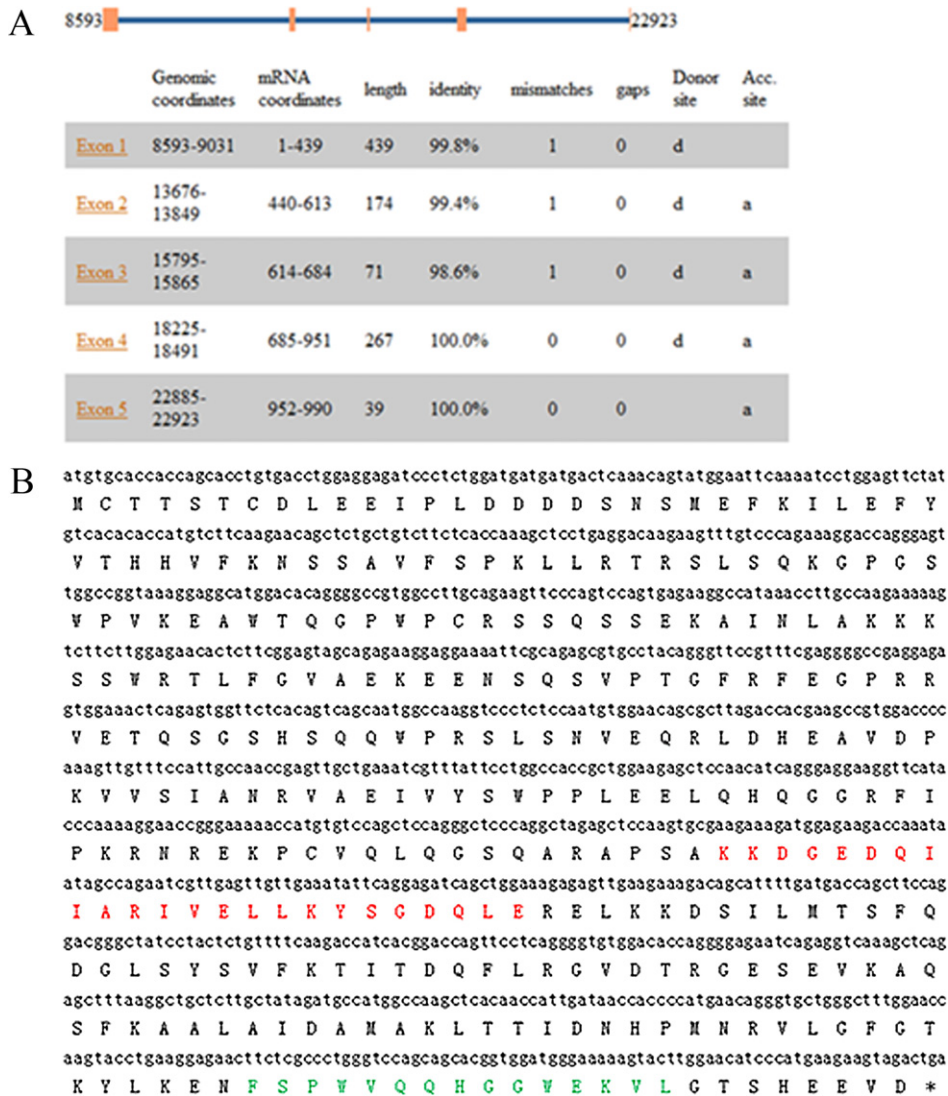


Fig. 1. Structure of the pBCL-G gene. (A) The exon–intron structure. The genomic and mRNA components were shown in detail. Five exons were found in the ORF. (B) Nucleotide and deduced protein sequences of pBCL-G. The asterisk represents the stop codon and the BH3 and BH2 domains are indicated by red and green amino acid residues, respectively.

(lower left quadrant). Cells treated with H₂O₂ as controls were individually stained with annexin V-PE or 7-AAD to define the lower right and upper quadrants.

2.7. Statistical analysis

Means and standard deviations were calculated using Microsoft Excel. Statistical analysis was performed with SPSS 17.0. Differences between two groups were assessed using an unpaired Student t test and two-tailed distribution, with *p* below 0.05 considered statistically significant.

3. Results and discussion

3.1. Analysis of basic characteristics of porcine BCL-G

The full-length cDNA of pBCL-G revealed one transcript containing an ORF covering 990 bp and yielding

a protein of 329 amino acids, with a molecular weight of about 37.19 kDa and a calculated isoelectric point of 7.10. We performed a Blast search in the pig reference genomic sequence database (Scrofa10) using the pBCL-G cDNA sequence (GenBank ID: JN628029) to determine the exon–intron structure of the pBCL-G gene. A 14,331 bp sequence covering the entire pBCL-G gene was retrieved (GenBank ID: 333785884). The alignment of genomic clones with the coding region of pBCL-G cDNA revealed that the exonic sequences were 99.7% matched. Exon–intron boundaries were initially estimated by aligning the coding region of pBCL-G cDNA against the amplified genomic sequences. This estimate revealed a total of 5 exons ranging from 39 to 439 nucleotides, and all of the observed splice acceptor and donor sites concurred with the GT-AG rule. The exon–intron structure is presented in Fig. 1A. The alignment of the protein sequence with the BLAST program (www.ncbi.nlm.nih.gov/BLAST) on the NCBI

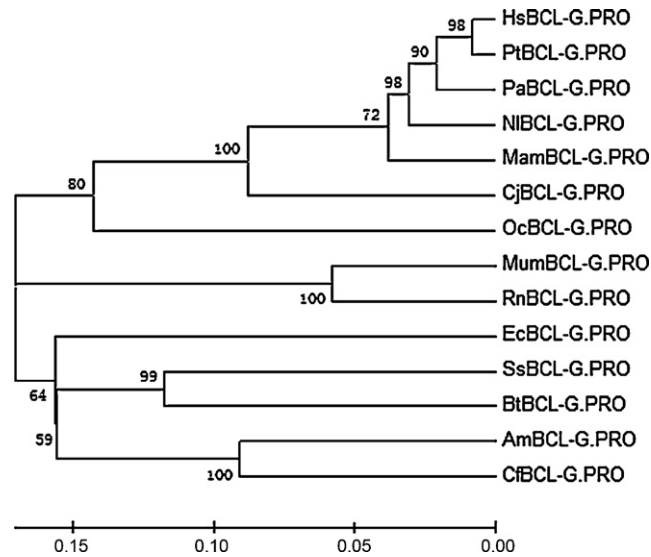


Fig. 2. Evolutionary relationships of porcine BCL-G, and other BCL-G proteins. The phylogenetic tree was drawn using MEGA4.0 software and the neighbor-joining method. The node points represent the percentage of node points in 500 bootstraps values. The abbreviations for species acronyms and corresponding GenBank accession numbers of the sequences used for construction of the phylogenetic tree are as follows: Am, *Ailuropoda melanoleuca*, XP.002924875; Bt, *Bos taurus*, NP.001015544; Cf, *Canis familiaris*, XP.534887; Cj, *Callithrix jacchus*, XP.002752184; Ec, *Equus caballus*, XP.001501335; Hs, *Homo sapiens*, NP.620048; Mam, *Macaca mulatta*, XP.001083791; Mum, *Mus musculus*, AAH25541; Ni, *Nomascus leucogenys*, XP.003265555; Oc, *Oryctolagus cuniculus*, XP.002712712; Pa, *Pongo abelii*, XP.002822986; Pt, *Pan troglodytes*, XP.001152279; Rn, *Rattus norvegicus*, NP.001019509; and Ss, *Sus scrofa*, JN628029.

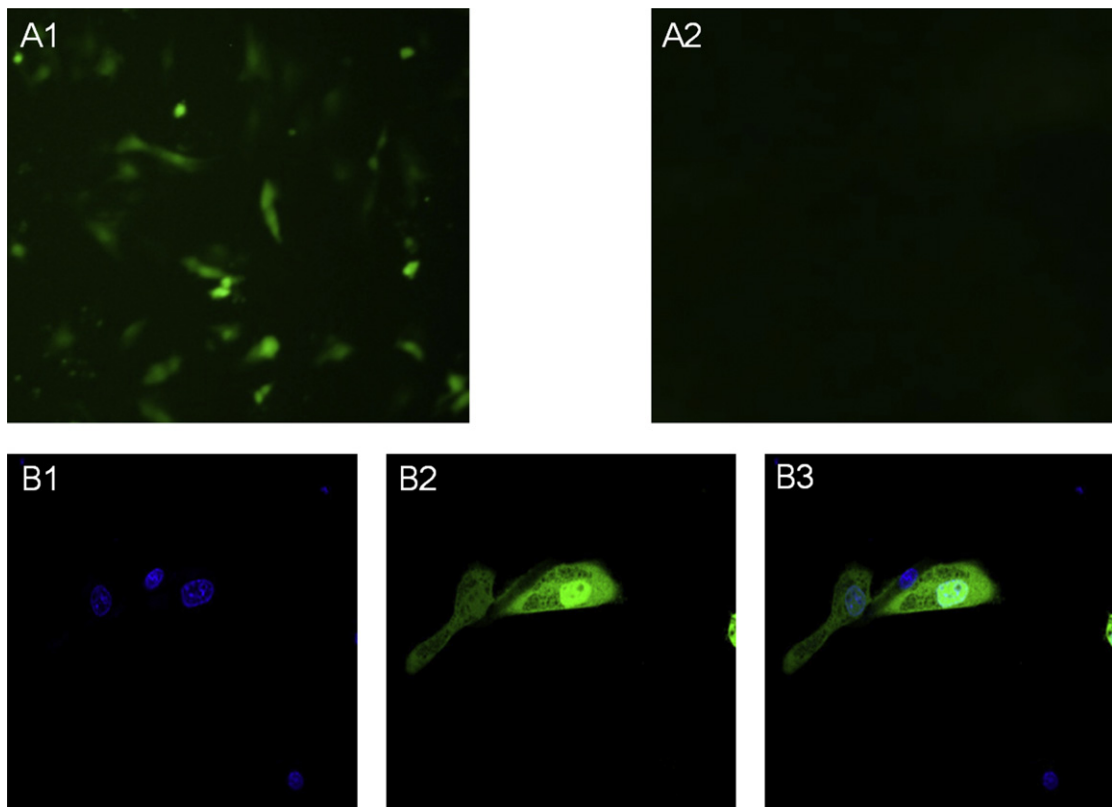


Fig. 3. Fluorescence analysis of expression of the GFP-pBCL-G fusion protein and its subcellular distribution in PK-15 cells. Recombinant plasmids were transferred into PK-15 cells using Lipofectamine 2000. The expression of GFP-pBCL-G was analyzed using inverted fluorescence microscope. (A1) PK-15 cells transfected with pEGFP-pBCL-G and (A2) PK-15 cells. Subcellular distribution of GFP-pBCL-G protein was analyzed by confocal microscopy. (B1) The nuclei were stained with Hoechst 33342. (B2) Green fluorescence protein. (B3) Overlaid images were produced by merging two signals together. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

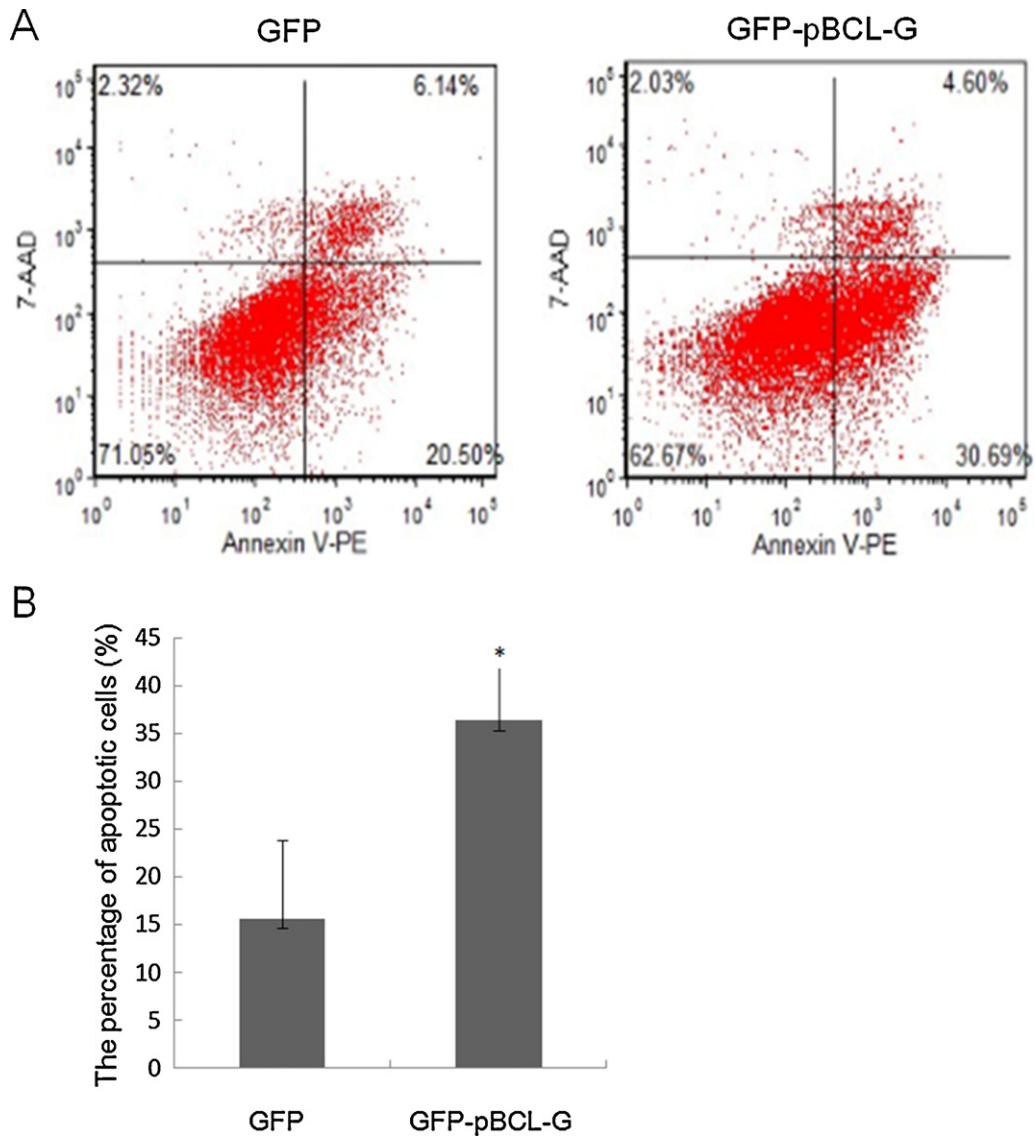


Fig. 4. Induction of apoptosis by poly I:C. Quantification of apoptosis by annexin V-PE/7-AAD staining and flow cytometry. (A) Representative FACS diagrams of PK-15 cells expressing GFP-pBCL-G or GFP treated with poly I:C for 18 h at 4 μ g/mL. (B) Quantification of annexin V-positive cells. Data are expressed as means \pm SD and the experiments were repeated three times at least and had statistical significance (* $p < 0.05$).

website showed that pBCL-G gene shares 75%, 73%, 74%, 71%, 72%, 71%, and 69% identities with homologous genes of *Bos taurus*, *Macaca mulatta*, *Ailuropoda melanoleuca*, *Pan troglodytes*, *Pongo abelii*, *Homo sapiens*, and *Mus musculus*, respectively. Domain prediction showed that pBCL-G protein may be a member of the Bcl-2 family because it contains the BH2 and BH3 domains of the Bcl-2 family (Fig. 1B) (Chittenden et al., 1995; Yin et al., 1994).

3.2. Phylogenetic analysis

We constructed a phylogenetic tree using MEGA4 software to evaluate the evolutionary relationship of pBCL-G with homologs from other species. BCL-G protein sequences from 14 different species, including human, chimpanzee, orangutan, gibbon, rhesus, rabbit, mouse,

rat, horse, pig, cattle, giant panda, and dog, were used for the unrooted phylogenetic tree constructed by the neighbor-joining method (Fig. 2). Phylogenetic analysis demonstrated that the phylogeny of pBCL-G is more related to cattle BCL-G than to human BCL-G. Pig, mouse, and human BCL-G proteins are located in three different clades, which indicate that BCL-G may function differently in these species.

3.3. Subcellular distribution of the GFP-pBCL-G fusion protein in PK-15 cells

The cDNA was subcloned into the pEGFP-C1 plasmid to generate the recombinant expressing plasmid pEGFP-pBCL-G, identify whether or not pBCL-G acts as a pro-apoptotic factor, and characterize the subcellular

localization of the pBCL-G protein. PK-15 cells were transiently transfected with the pEGFP-pBCL-G plasmid or pEGFP-C1 as a negative control. Fluorescence analysis showed that GFP-pBCL-G proteins were expressed after transfection (Fig. 3A). Furthermore, fluorescence signals were examined by confocal fluorescence microscopy. Fusion proteins were found to be distributed in both the nucleus and the cytoplasm (Fig. 3B).

3.4. Analysis of induced apoptosis

pBCL-G contains the BH3 domain, which is a key structure for the apoptotic function of pro-apoptotic factors of the Bcl-2 family (Reed, 2008; Strasser, 2005). We applied annexin V-PE/7-AAD staining in combination with flow cytometry to differentiate between apoptosis and necrotic cell death and explore the role of pBCL-G. After transfection with pEGFP-pBCL-G and pEGFP-C1 vectors, PK-15 cells were incubated with 4 μ g/mL poly I:C for 18 h and then co-incubated with PE-labeled annexin V as an early marker for phosphatidylserine externalization at the cell membrane. Early apoptotic events are located in the lower right quadrant of the fluorescence activated cell sorting (FACS) diagrams, whereas late apoptosis is found in the upper right quadrant (Fig. 4A). Early apoptotic events revealed a significant increase in apoptosis when cells expressed GFP-pBCL-G (Fig. 4B) compared with cells expressing GFP control. pBCL-G may thus be a pro-apoptotic factor involved in the immune response against viral infection.

In conclusion, the present work described the features of the nucleic acid and deduced amino acid sequences of pBCL-G, investigated its subcellular distribution, and verified its pro-apoptotic function. The fundamental data accumulated in this study serves as a foundation for understanding the mechanism of the pro-apoptotic action of pBCL-G and provides a potential medical model for investigating diseases related to human BCL-G.

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