The apoptosis-associated protein BNIPL interacts with two cell proliferation-related proteins, MIF and GFER

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Abstract Bcl-2/adenovirus E1B 19 kDa interacting protein 2-like, BNIP-2-like (BNIPL) is a recently cloned and characterized apoptosis-associated protein that shares 72% homology with BNIP-2. It is highly expressed in human placenta and lung. A yeast two-hybrid system was used to obtain two BNIPL-interacting proteins, MIF (macrophage migration inhibitory factor) and GFER (growth factor erv1 (*Saccharomyces cerevisiae*)-like). The interactions were confirmed by glutathione *S*-transferase pull-down assay in vitro and co-immunoprecipitation assay in vivo. Colony formation assay and cell proliferation test suggest that overexpression of BNIPL could inhibit the growth of BEL-7402 cells. These findings suggest that BNIPL may physically bind to cell proliferation-related proteins, MIF and GFER.

Key words: *BNIPL*; Yeast two-hybrid; Cell proliferation; Apoptosis

1. Introduction

Cell proliferation and apoptosis are the two essential opposite cellular processes [1]. Apoptosis, programmed cell death, is a regulated process of selective cell deletion while the proliferation is one of the choices for the survival cells [2]. Both of the processes are physiologically closely associated and regulated. Their coordination and balance are crucial for governing both homeostasis and normal development of cells and tissues. Deregulated cell proliferation and suppressed apoptosis constitute the minimal common platform upon which cancer and autoimmune diseases occur [3–5]. The signal transduction between the proliferation and cell death has been recognized with many observations. The mutations promoting inappropriate entry into cell proliferation often promote apoptosis [1]. The overexpression of anti-apoptotic members of the Bcl-2 family proteins can suppress proliferation and promote entry into G_0 [6]. Also, the existing experimental data suggest that there would be a functional relation between the proliferation and death pathway for preventing the survival and expansion of clones of aberrant cells [1]. A convincing example is that a controller of cell proliferation, Myc, was found to be a powerful inducer of apoptosis [3]. Most recently, the death receptors that are regarded as signal of apoptosis conventionally or their downstream regulators are reported to promote cell proliferation [7]. Therefore, it will become necessary to find out potential cross linkers.

Bcl-2/adenovirus E1B 19 kDa interacting protein 2-like, BNIP-2-like (BNIPL) is a recently cloned and characterized apoptosis-associated protein that shares 72% homology (46% identity) with BNIP-2. BNIP-2 can interact with the Bcl-2 and adenovirus E1B 19 kDa protein and has the function of protecting cells against death induced by viral infection or other proapoptotic stimuli [8]. It has been reported that BNIPL can cause apoptosis mediated by an apoptosis-inducing BCH (BNIP-2 and Cdc42GAP homology) domain [9–11].

We focus on the cell functions of this apoptosis-associated protein BNIPL. A yeast two-hybrid screening is carried out to find out the interacting proteins of BNIPL. The effect of BNIPL on the growth of tumor cells is also investigated. Here, we report the experimental results that support another role for BNIPL in the cytoplasm.

2. Materials and methods

2.1. Northern blot analysis

Northern blot analysis was carried out using Human 8-lane MTN Blot membrane (Clontech) as per manufacturer's protocol. The *BNIPL* (accession no. AF193056) cDNA probe was prepared by the random primer labeled with ^{32}P using rediprime II random prime labeling system (Amersham Pharmacia Biotech). After prehybridization, hybridization and highly stringent washing, the membrane was exposed to an X-ray film at −70°C.

2.2. Yeast two-hybrid screening

A LexA-based yeast two-hybrid system DupLEX-A (OriGene, Rockville, MD, USA) was used in library screening. The complete coding sequence of BNIPL was cloned into the vector pEG202-NLS containing a DNA-binding domain as the bait. In the screening, the resulting plasmid and human lung cDNA library fused to activation domain were sequentially transformed into the yeast strain EGY48 that harbors the *LEU2* and *lacZ* report genes under the control of LexA binding sites. The total 3×10^6 transformants were screened for the positive clones with galatose-dependent $lacZ^+$ and $LEU2^+$ phenotypes by replica plating on galactose/raffinose media lacking glucose, uracil, histidine and tryptophane in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside or in absence of leucine. A mating

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Abbreviations: BNIPL, Bcl-2/adenovirus E1B 19 kDa interacting protein 2-like; BCH, BNIP-2 and Cdc42GAP homology; MIF, macrophage migration inhibitory factor; GFER, growth factor erv1 (*Saccharomyces cerevisiae*)-like; GST, glutathione *S*-transferase; HA, hemagglutinin; HRP, horseradish peroxidase

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test was performed to pluck the specific protein–protein interactions. The pJG4-5-cDNA constructs were isolated from the positive yeast clones and sequenced. Homology was searched with the BLAST algorithm through the National Center for Biotechnology Information web site at http://www.ncbi.nlm.nih.gov.

2.3. Glutathione S-transferase (GST) pull-down assay

The DNAs encoding the entire protein sequences of MIF (macrophage migration inhibitory factor) and GFER (growth factor erv1 (*Saccharomyces cerevisiae*)-like) were cloned into the pGEX-5X-1 vector (Amersham Pharmacia Biotech) that has GST as fusion partner, respectively. The GST fusion proteins were expressed in 0.1 mM isopropyl-1-thio-β-D-galactopyranoside-induced *Escherichia coli* strain BL21 (DE3) and purified by glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech). The *BNIPL* was cloned into the vector pCMV-HA2. The hemagglutinin (HA)–BNIPL fusion protein was generated by the TNT T7 Quick Coupled Transcription/Translation System (Promega) and tested by Western blotting with anti-HA antibody (Clontech). Equivalent amounts of GST–MIF, GST–GFER and GST alone immobilized on glutathione–Sepharose 4B beads as well as 5 μl HA-tagged BNIPL were incubated for 2 h at 4°C with gentle shaking in 50 μl NETN buffer consisting of 100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 7.5, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride. The beads were washed three times in 100 μl buffer H with the components of 20 mM HEPES, pH 7.7, 50 mM KCl, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40 and 0.007% (v/v) β-mercaptoethanol, and boiled in 20 μl elution buffer. Proteins in the supernatant were separated by 12% SDS–PAGE. The separated proteins were transferred to PVDF membrane and immunoblotted with rabbit anti-HA (Clontech) primary antibody and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Calbiochem). The HA-tagged protein was visualized with DAB substrate kit (Roche) for HRP.

2.4. Co-immunoprecipitation assay

COS-7 (monkey kidney) cells were cultivated in 6-well plates to 70% confluence and co-transfected with 2 μg plasmids of HA2–BNIPL with Myc-tagged MIF and Myc-tagged GFER, respectively. After 48 h of incubation in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies) under the condition of 5% CO_2 at 37°C, the cells were harvested and washed twice with ice-cold PBS and lysed with lysis buffer (supplied in Luciferase Detection Kit, Roche). In each precipitation reaction, 500 μl lysate was pre-cleaned with protein A/G PLUS agarose (Santa Cruz Biotechnology) for 1 h at 4°C with gentle shaking followed by immunoprecipitation with 200 ng monoclonal anti-HA antibody (Santa Cruz Biotechnology) at 4°C overnight. The pellets were washed with 100 μl lysis buffer three times. The precipitated proteins were eluted from the beads with protein loading buffer, and separated by 15% SDS–PAGE. Proteins were transferred to PVDF membrane and immunoblotted with mouse anti-Myc primary antibody (Invitrogen) and HRP conjugated rabbit anti-mouse IgG. The immunocomplexes were visualized with SuperSignal West Femto Maximum sensitivity substrate for HRP (Pierce).

2.5. Colony formation assay

BNIPL-encoding sequence was cloned into the expression vector pcDNA4-HisMax-TOPO (Invitrogen). The plasmid DNA was extracted and purified with the plasmid purification system (Qiagen). To follow the procedures recommended by the manufacturer (Life Technologies), BEL-7402 cells (human epithelial-like hepatoma) cultivated in a 6-well plate were transfected with pcDNA4-HisMax/BNIPL, pcDNA4-HisMax/p53 and pcDNA4-HisMax void vector by means of LipofectAMINE, respectively. The transfected cells were incubated for 14 days under the condition of 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum (Life Technologies) and 200 μg/ml zeocin. The colonies were examined with an inverted microscope and assigned scores. This kind of experiment was repeated three times.

2.6. Effect of BNIPL on the proliferation of BEL-7402 cells

BEL-7402 cells were cultivated on four 96-well plates with 1×10^4 cells per well. Under the experimental conditions recommended by manuacturer (Life Technologies), these cells were transfected with plasmids pcDNA4-HisMax/BNIPL, pcDNA4-HisMax/p53 and pcDNA4-HisMax void vector by means of LipofectAMINE, respectively. The transfected cells were supplied with DMEM supplemented with 10% fetal bovine serum (Life Technologies) and 200 μg/ml zeocin, and incubated under the condition of 5% CO_2 at 37°C for 1 week. The cells were counted every 24 h to detect the proliferation of cells in the following 4 days. Every set of data is an average over the 6 wells.

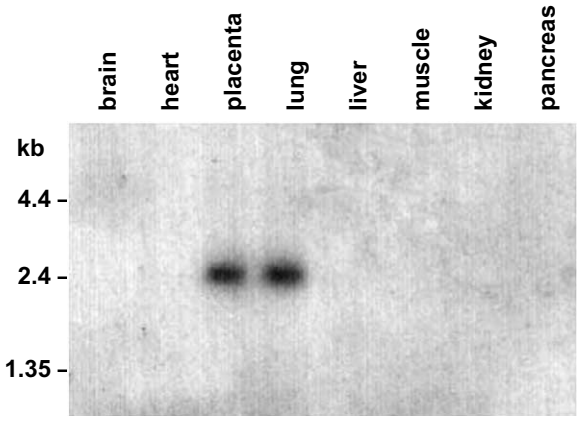

Fig. 1. Expression patterns of *BNIPL* mRNA in different human normal tissues. The labeled *BNIPL* cDNA as a probe hybridized with a multiple-tissue Northern blot (Clontech) containing 2 μg poly(A) RNA from different human tissues in each lane.

3. Results

3.1. Tissue expression pattern of BNIPL

The tissue expression pattern of *BNIPL* (accession no. AF193056) was investigated by hybridizing its cDNA probe to a multiple-tissue Northern blot. A transcript of approximately 2.4 kb was observed in human placenta and lung, shown in Fig. 1. The expression of *BNIPL* was investigated in various cell lines, including NVI-H446 (human small cell lung cancer), SMMC-7721 (human epithelial-like liver cancer) and BEL-7402 (human epithelial-like hepatoma). The expression levels in the three cancer cell lines have no detectable differences (data not shown).

3.2. Identification of BNIPL-interacting proteins through yeast two-hybrid screening

To identify proteins binding to BNIPL, we screened a human lung cDNA library in a yeast two-hybrid assay using BNIPL as the bait. A screen of approximately 1.5×10^6 yeast transformants yielded 33 positive colonies that grew on the selective medium and expressed reporter genes. Mating tests were conducted to pluck the specific interactions. Sequence analysis in GenBank database revealed that 16 of the 33 clones encoded 10 proteins with known function and two proteins with unknown function (data not shown). Among all the positive colonies obtained from yeast two-hybrid screening, MIF and GFER (augmenter of liver regeneration) appeared most frequently. In addition, the obtained positive clones have the entire coding sequences in correct open reading frames.

3.3. BNIPL specifically binds to MIF or GFER

A GST pull-down assay in vitro was carried out to verify the interactions observed in the yeast two-hybrid screening. MIF and GFER were fused to GST and expressed in *E. coli* strain BL21 with the molecular weight of about 43 and 45 kDa, respectively. The fusion partner GST was also expressed

in the strain BL21 with the molecular weight of 29 kDa as a control for the specificity of the bindings (Fig. 2A). The bacterial expressed GST–MIF and GST–GFER conjugated to glutathione–Sepharose beads were incubated with HA-tagged BNIPL translated in vitro, respectively. The HA–BNIPL protein appeared as a 35 kDa band in translated mixture. After stringently washing, the bound proteins were eluted by elution buffer. The complexes were analyzed by SDS–PAGE and Western blotting using anti-HA antibody. BNIPL was detected to exist on immunoblot when incubated with two GST fusion proteins, respectively, but not with GST alone (Fig. 2B). The GST pull-down assay shows that BNIPL specifically interacts with MIF and GFER, respectively.

The co-immunoprecipitation approach was used to confirm the interactions in mammalian cells. COS-7 cells were cotransfected with HA-tagged BNIPL and Myc-tagged MIF and GFER, respectively. pCMV-HA2 and pCMV-Myc vectors were used as control. The HA-tagged BNIPL was about 35 kDa. The Myc-tagged fusion proteins were about 16 and 18 kDa, respectively (Fig. 2C). Anti-HA antibody and protein A/G-agarose were added into the cell lysates so as to precipitate HA-tagged BNIPL. Two Myc-tagged proteins were detected in the precipitate by Western blotting using anti-Myc antibody (Fig. 2D). Since the anti-HA antibody and anti-Myc antibody were both monoclonal antibodies, we also detected the heavy chain and light chain of mouse IgG besides the Myc-tagged protein. Both the results of in vitro and in vivo binding assays are in agreement with those of the yeast two-hybrid assay.

Both MIF and GFER are cytokines generated by immune or non-immune cells. They are distributed in both intercellular and intracellular spaces in vivo. The subcellular localizations of overexpressed MIF and GFER in COS-7 showed that both the proteins localized in the cytoplasm, which was consistent with the subcellular localization of BNIPL [11]. Also, this suggests that the interactions between BNIPL and MIF or GFER could be in the cytoplasm.

3.4. *The overexpression of BNIPL inhibits the growth of BEL-7402 cells*

The results of yeast two-hybrid screening indicate that BNIPL may be involved in cell growth. To determine the effects of BNIPL on the cell proliferation, the colony-forming efficiency and the growth curve of BNIPL-transfected BEL-7402 cells were measured using p53 as a positive control. The overexpression of BNIPL in BEL-7402 cells not only reduced

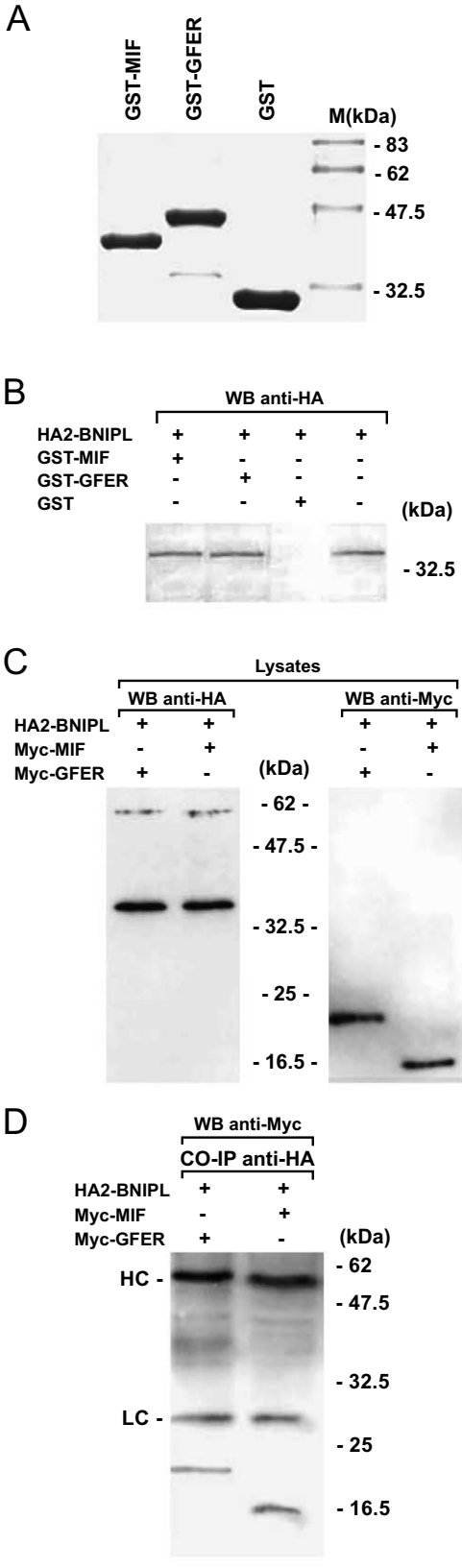

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Fig. 2. Validation of binding of BNIPL with MIF or GFER. A: Fusion proteins of GST-MIF, GST-GFER and GST were expressed in *E. coli* BL21, respectively. After purification using glutathione–Sepharose 4B beads, the expressed fusion proteins were analyzed by SDS–PAGE and stained by Coomassie. B: The interaction of BNIPL with MIF or GFER was analyzed by GST pull-down assay in vitro. GST fusion proteins immobilized on glutathione–Sepharose 4B beads were incubated with HA2–BNIPL translated in vitro. Interacting proteins were immunoblotted with rabbit anti-HA primary antibody. BNIPL binds specifically to MIF and GFER, but not to GST alone. The translation product of *BNIPL* in vitro was used as a control. C: Expressions of HA–BNIPL and Myc–MIF or Myc–GFER in co-transfected COS-7 were analyzed by Western blotting with mouse anti-HA or anti-Myc primary antibody. D: The specificity of interactions between BNIPL and MIF or GFER was confirmed by co-immunoprecipitation in vivo. Cell lysates from COS-7 cells co-transfected with HA–BNIPL and Myc–MIF or Myc–GFER were immunoprecipitated using mouse anti-HA antibody. The precipitated proteins were eluted from the protein A/G PLUS agarose with protein loading buffer, and separated by SDS–PAGE and immunoblotted with mouse anti-Myc primary antibody. HC and LC represent the heavy chain and light chain of the mouse IgG.

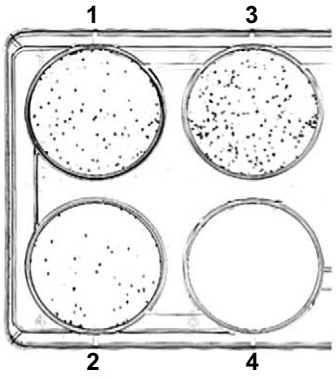

Fig. 3. The effect of BNIPL on the colony formation of BEL-7402 cells. The transfected cells were incubated in DMEM supplemented with 10% fetal bovine serum and 200 μg/ml zeocin for 2 weeks. Zeocin-resistant colonies were then examined with an inverted microscope and assigned scores. p53 and void vector were positive and negative control, respectively. 1: pcDNA4-HisMax/BNIPL; 2: pcDNA4-HisMax/p53; 3: void vector; 4: not transfected.

the numbers of colonies (Fig. 3) but also inhibited the cell growth speed. The growth rate of the BEL-7402 cells transfected with BNIPL was reduced by about 30% in contrast to the reduction of 80% by p53 (Fig. 4). The two results clearly indicate that the transfection of the pcDNA4-HisMax plasmid containing *BNIPL* into human BEL-7402 cells is able to suppress the cell growth.

4. Discussion

The yeast two-hybrid system has been a potent tool for identifying binding partners of protein of interest to study its potential role in biological activities in vivo. We isolated two proteins interacting with BNIPL, MIF, a famous pleiotropic protein in inflammatory and immune responses, and GFER. The bona fide interactions were further confirmed in vitro and in vivo.

One partner of BNIPL, MIF, was one of the earliest discovered cytokines four decades ago [12,13]. MIF is identified as an integral regulator not only of the inflammatory and immune response [14], but also in growth factor-dependent cell proliferation, cell cycle, angiogenesis and tumorigenesis [15]. The BNIPL–MIF interaction may occur following uptake of MIF into target cells by non-receptor-mediated pathway, which is one of the three hypothetic independent signal mechanisms for MIF [16]. The inhibition of secreted MIF by anti-MIF antibody prevents cell growth during serum-induced cell cycle progression [17]. Other studies also suggest that increased cytosolic MIF expression in tumors is related to the proliferative properties of tumor cells [16]. The interaction between BNIPL and MIF suggested that BNIPL would be involved in governing cell proliferation, the known biological activities of MIF. Our experiment showed the overexpression of BNIPL in BEL-7402 cells suppresses cell growth. This suggested that BNIPL could inhibit MIF-mediated tumor cells proliferation.

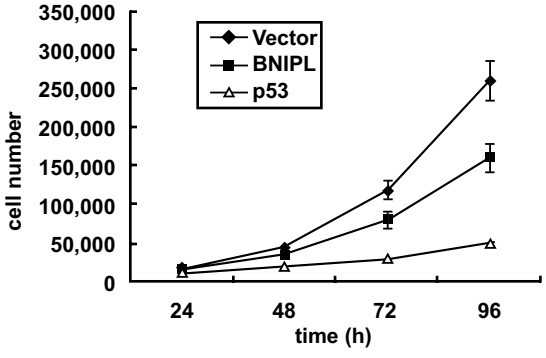

Fig. 4. The effect of BNIPL on the growth of BEL-7402 cells. The transfected cells were incubated in DMEM supplemented with 10% fetal bovine serum and 200 μg/ml zeocin for 1 week. The numbers of zeocin-resistant cells were counted every day in the following 4 days. The numbers represent the average of 6 wells. Error bars indicate the standard deviations. p53 and void vector were positive and negative control, respectively.

Another BNIPL-interacting protein, GFER, acts as an augmenter of liver regeneration. The interaction between BNIPL and GFER also hints that BNIPL functions in cell proliferation. GFER triggers liver regeneration by activation of inactive transcription factors that pre-exist in the liver [18]. The binding of BNIPL with GFER could affect the function of the endogenous growth factor and therefore inhibit the growth of liver cells. The expression of BNIPL was detected in two cell lines but not normal liver tissues. The interaction with GFER and the specific expression pattern suggest that expression of BNIPL may respond to hepatectomy or toxic damage.

The bindings of BNIPL with two endogenous cytokines, MIF and GFER, indicated that BNIPL is functional in efficient regulation of cell proliferation. According to bioinformatics analysis, it should be noted that BNIPL has an apoptosis-inducing BCH domain and several biochemical and morphological assays also indicate that BNIPL could induce extensive morphological changes and cell death [9–11]. However, BNIPL-induced apoptosis did not directly involve in the known caspases and the Bcl-2/Bcl-xL inhibition [11]. BNIPL could induce apoptosis by itself. The interactions between BNIPL and MIF or GFER suggest that BNIPL would be involved in regulation of cell proliferation mediated by the two cytokines, while MIF and GFER could also take part in the apoptosis induced by BNIPL. The cell proliferation and apoptosis are two closely associated and regulated physiological activities [5]. Cells of damaged tissues have to be confronted with only one choice of survival or death. For our current best understanding, the interactions of BNIPL with the two proteins would provide a mechanism for maintaining in vivo homeostasis between cell proliferation and apoptosis.

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