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Research Article

A putative role for human BFK in DNA damage-induced apoptosis

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Human BFK (BCL-2 family kin) is a novel pro-apoptotic BCL-2 family member specifically expressed in the gastrointestinal tract. BFK has the characteristic BH3 domain, which was shown to be essential for the apoptosis-inducing activity of pro-apoptotic BCL-2 family members. When overexpressed, BFK interacts with BCL-XL and BCL-W but not BCL-2 or BAD in co-immunoprecipitations studies. We find that BFK exhibits striking similarity to BID in the way it is activated through cleavage during apoptosis. The endogenous and cleaved versions of BFK are readily recognized by the rabbit and mouse sera raised against human BFK. An ideal caspase 3 or 7 target sequence, DEVD (amino acids 38–41), is evident N-terminal to the BH3 domain. A recombinant version of the protein containing all residues downstream of the putative caspase cleavage site induces apoptosis in human colon cancer cells, HCT116, and in wild-type mouse embryonic fibroblasts (MEFs), which can be reversed by co-expression of BCL-XL or BCL-W. BFK becomes activated through caspase-dependent cleavage during DNA damage-induced apoptosis. The cleaved form of the protein is dependent on the presence of BAX or BAK for its ability to induce apoptosis, since $BAX^{-/-}$-$BAK^{-/-}$ double-knockout MEFs are completely resistant to BFK-induced apoptosis.

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1 Introduction

The importance of BCL-2 family members in human malignancy is evident by the frequency of mutations documented in different cancer types. For example, BCL-2 is overexpressed in the majority of human follicular lymphoma, due to translocation t(14;18) placing the BCL-2 gene next to the immunoglobulin (Ig) enhancer region [1]. BAX was found mutated in 50% of human colon carcinomas with defects in mismatch repair genes [2]. BCL-2 family members are proteins with critical regulatory functions in various cell death induction pathways [3]. Anti-apoptotic members such as BCL-2, BCL-XL, BCL-W and MCL-1 need to be neutralized by other BH3-domain family members, such as BID, BAD, BIM, PUMA or NOXA, before the pro-apoptotic BAX and BAK can destabilize mitochondrial or endoplasmic reticulum (ER) membranes, initiating downstream apoptotic signaling cascades [4, 5].

BCL-2 family kin (BFK) is a recently identified member of the BCL-2 family and appears to have a weak ability to induce apoptosis. The initial study by Coultas *et al.* [6] reported an interesting expression pattern restricted to the mice mammary tissue and uterus. Human BFK (hBFK) and mouse BFK share 69% sequence identity. Human BFK is a 163-amino acid-long member of the BCL-2 family lo-

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Abbreviations: ER, endoplasmic reticulum; **hBFK**, human BCL-2 family kin; **MEF**, mouse embryonic fibroblast; **t-hBFK**, truncated hBFK; **wt**, wild type

calized to chromosome 1p13.1 and carries the characteristic BH2 and BH3 domains [6]. hBFK also has a pseudo-BH1 domain in which some, but not all, of the critical amino acids are conserved. Examining the sequence of hBFK, a DEVD sequence at amino acids 38–41 is evident in the human protein but not in the mouse version, which has a DEPC instead [6]. The DEVD tetrapeptide is the ideal target sequence for caspases 3 and 7 cleavage. This difference suggests that the human protein may have acquired novel function(s) during evolution. hBFK was reported to be expressed predominantly in the human gastrointestinal system [7]. Interestingly, BFK expression was shown to decrease during malignant transformation in colon cancer samples [7]. More recently, high levels of mouse BFK expression, influenced by androgens, have been reported in the principal cells of the epididymis and a role for mBFK in the differentiation of the initial segment of the epididymis was suggested [8].

We have cloned the BFK gene independently and have concentrated our studies on the physiological function of the human BFK protein. At present, some data exist regarding the expression of BCL-2, BAX, BID and BH3-only proteins, such as NOXA and PUMA, in certain segments of the gastrointestinal tract [9], but the mechanisms by which BCL-2 proteins regulate spontaneous or damage-induced apoptosis are not yet clear. Initially BAX [10] and later the BH3-only members NOXA [11, 12], PUMA [13] and BID [14] were shown to be regulated in a p53-dependent manner in response to DNA damage. It is clear that p53 utilizes pro-apoptotic BCL-2 family proteins to induce apoptosis; however, which ones are critical in the intestine and/or other tissues needs to be elucidated.

Here we report that human BFK interacts with BCL-XL and BCL-W, but not with BCL-2 or BAD. Furthermore, we found that the hBFK protein becomes cleaved in a caspase-dependent manner during DNA damage-induced apoptosis. Full-length hBFK is incapable of inducing apoptosis in HEK 293T cell or colon cancer cells, such as HCT116 or SW480. However, a truncated version (t-hBFK) corresponding to the part of the protein downstream of the caspase 3 or 7 cleavage site induces apoptosis in HCT116 or wild-type (wt) mouse embryonic fibroblasts (MEFs). The pro-apoptotic activity of t-hBFK is completely dependent of the presence of BAX or BAK since double-knockout MEFs were resistant to killing. Co-expression of BCL-XL or BCL-W reverses the killing activity of t-hBFK in both HCT116 and MEFs.

2 Materials and methods

2.1 Cell lines

Human embryonic kidney fibroblast cells transformed with T antigen (HEK293T) and human colon cancer cells SW480 were obtained from ATCC (Manassas, VA, USA) and grown in DMEM. Human colon cancer cells HCT116, grown in RPMI 1640, were a kind gift from Dr. Wafik El-Deiry (University of Pennsylvania Medical School, Philadelphia, PA, USA) and wt and $Bax^{-/-}$-$Bak^{-/-}$ double-knockout MEFs, grown in DMEM, were a kind gift from Dr. Stanley Korsmeyer (Harvard University, Boston, MA, USA). All media were supplemented with FBS (10%), L-glutamine (1%) and penicillin/streptomycin (1%). Cells were grown at 37°C, in 5% CO_2 incubators. All media were purchased from Gibco-BRL.

2.2 Plasmid constructs

The EST for human BFK (I.M.A.G.E.:1161036) was purchased from ResGen-Invitrogen Corp. (Huntsville, AL, USA). After sequencing the cDNA insert, we established the presence of the start and stop codons, which suggested the EST contained the complete coding sequences of hBFK. A non-tagged version of hBFK cDNA was cloned into the *Eco*RI site of pcDNA3. We amplified the hBFK cDNA from the pT7T3-PAC using primers that provided an *Xba*I cleavage site upstream of the second codon and an *Eco*RI cleavage site after the stop codon. After appropriate digestion of the PCR insert and modified versions of pcDNA3 sequence (modified multiple cloning site sequence available upon request), we created N-terminal, in-frame fusion of FLAG, HA and MYC tags to the hBFK cDNA; these constructs were named: pcDNA3-FLAG-hBFK, pcDNA3-HA-hBFK, pcDNA3-MYC-hBFK respectively. Tagged versions of t-hBFK were generated by cloning cDNA sequences downstream of the putative caspase 3 or 7 cleavage site (bases 120–492) into the *Xba*I- and *Apa*I-digested modified pcDNA3-FLAG, pcDNA3-HA and pcDNA3-MYC. This step provides an in-frame fusion of the tags with the inserted sequence. The ability of these vectors to express the protein of expected size was verified first through sequencing and then through overexpression in 293T cells and Western blotting using the corresponding antibodies against the tags. For protein purification purposes we generated an N-terminally 6×histidine-tagged version of hBFK. We excised cDNA from pcDNA3-FLAG-hBFK using the *Kpn*I/ACC65 I and *Eco*RI cutting sites and cloned the fusion sequences of

FLAG-hBFK in-frame to the 6×HIS sequences of the IPTG-inducible bacterial expression vector pET30a (+) (Novagen, Germany). The pcDNA3-FLAG-BCL-XL, pcDNA3-FLAG-BCL-2, pcDNA3-FLAG-BCL-W, pcDNA3-FLAG-BAD and pcDNA3-caspase9-MT-HA, pcDNA3-BID, pcDNA3-FLAG-tBID, pcDNA3-HA-tBID have been described previously [15–18]. All restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA) and used according to manufacturer's recommendations.

2.3 Transfection conditions

For co-immunoprecipitation purposes, 293T cells were plated into 10-cm^2 dishes at a density 5×10^6, 24 h before transfection. The calcium phosphate protocol was used to transfect 3 μg pcDNA3-FLAG-hBFK or pcDNA3-HA-hBFK, 1 μg pcDNA3-HA-BID, 2 μg pcDNA3-FLAG-BCL-XL or pcDNA3-FLAG-BCL-W, and 3–4 μg pcDNA3-FLAG-BCL-2 in different combinations. To protect cells from unwanted toxic effects of BCL-2 family members, mutant caspase 9 was co-expressed using 4 μg pcDNA3-caspase9^{MT}-HA or pcDNA3-caspase9^{MT}-MYC with each condition. The total plasmid DNA transfected was brought to 10 μg for each plate by the addition of empty vector (pcDNA3-FLAG or pcDNA3-HA).

For apoptosis assays using HCT116, the cells were plated at a density of 5×10^5 cells/well into 6-well-dishes 18 h ahead of time and transfection was carried out using Lipofectin as recommended by the manufacturer (Invitrogen). Control vector 4 μg pcDNA3-HA, 4 μg pcDNA3-hBFK, 2 or 4 μg pcDNA3-HA-delBFK, 2 μg pcDNA3-FLAF-BCL-XL and 2 μg pcDNA3-delBFK, 2 μg pcDNA3-FLAF-BCL-W and 2 μg pcDNA3-delBFK were transfected into the cells in triplicate wells. The total amount of plasmid DNA was brought to 2 μg using empty vector and 0.4 μg pEGFP-C2 was co-transfected to identify specifically the transfected cells during apoptosis assays. Cells were analyzed for apoptosis induction 24 h after transfection.

$BAX^{-/-}$-$BAK^{-/-}$ double-knockout or wt MEFs were plated into 12-well dishes at 10^5 cells/well 1 day before the Lipofectamine 2000 (Invitrogen) protocol was applied as recommended.

2.4 Apoptosis assays

After transfection, apoptosis in HCT116 cells was assessed by the proportion of annexin V-PE-staining, pEGFP-C2-positive cells divided by the total number of pEGFP-C2-positive cells. The number of fluorescently labeled cells was determined using flow cytometry (UMICH, Cancer Center Flow Core Facility, Ann Arbor, MI, USA). AnnexinV-PE staining was performed on live cells after trypsinization within 1 h after collection according to manufacturer's instruction (Biovision, Mountain View, CA, USA).

For the quantification of apoptosis in MEFs, we used the blue-cell-count morphological test. MEFs co-transfected with pCMV-BOS-βgal were fixed and stained with a 4% X-gal solution on the next day. The overall transfection efficiency was calculated using the control wells; four to five visual fields were scanned for each well and a total of 300 cells were counted using the light microscope.

2.5 Co-immunoprecipitation

HEK293T cells transfected with the different plasmid combinations as described above were collected after 24 h and lysed in 0.2% NP-40, 10 mM HEPES (pH 7.55), 5 mM $MgCl_2$, 1 mM EGTA, 142 mM KCl supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Cell debris was precipitated out, and the remaining supernatant was used for immunoprecipitation. A fraction (1/20) of the lysate was saved for analysis of the protein expression in the whole cell lysate. Sepharose-protein A/G (40 μL) and the following quantities of precipitating antibodies were added to the supernatant: 5 μg anti-HA (clone 12CA5) mAb (Boehringer Mannheim, Mannheim, Germany), 5 μg anti-FLAG (M6) mAb (Sigma-Aldrich, St. Louis, MO, USA) or 2 μg anti-MYC polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell lysate, Sepharose beads and the precipitating antibody were incubated overnight on a 4°C rotating shaker. The beads were precipitated and washed five times in the 0.2% NP-40 buffer described above. After the final wash, 40 μg 2XSDS-loading buffer was added to the beads, which were boiled for 5 min. Co-immunoprecipitated proteins were analyzed by Western blotting analysis.

2.6 Immunoprecipitation of endogenous hBFK

To detect the endogenous hBFK cleavage product in SW480 colon cancer cells after DNA damage induction, cells were lysed in 0.2% NP-40 buffer (as defined above) and 10 μL serum from rabbit 886 (from bleed 5) was used for immunoprecipitation. For the negative control 15 μL serum from the rabbit pre-injection bleed was used.

2.7 Production of anti-hBFK mono- and polyclonal antibodies

Recombinant 6×HIS-hBFK [see above for the cloning steps of the bacterial inducible expression vector pET30 (a+)-6×HIS-hBFK] was produced in large quantities in BL21Star(DE3)pLysS *E. coli* after IPTG induction. Nickel column purification was carried out using the His·Bind Kit (Novagen, Germany) to purify the 6×HIS-hBFK according to manufacturer's instructions. Among the eluted fractions there were two with greater than 85% purity for the recombinant 6×HIS-hBFK. These fractions were subjected to SDS-PAGE electrophoresis; the gels were stained with Coomassie Blue and the correct size band was excised from the gel and sent to Lampire Biological Laboratory (Pipersville, PA, USA). Approximately 300 μg protein/injection was used to inject two rabbits (nos. 886, 887) five times (Expressline Protocol was extended). 'Prebleed' serum was obtained before the first injection and used for control purposes later. The first injection was given with complete Freund's adjuvant and the remaining with incomplete Freund's adjuvant.

For the production of mAbs, recombinant protein was purified in the same manner. In this case the fractions containing 6×HIS-hBFK were dialyzed against excess PBS at 4°C, and mice were injected four times with 20–25 μg recombinant protein/injection. Immunization and fusion protocols were performed at the University of Michigan Hybridoma Core Facility following established standard procedures. Hybridoma supernatants were collected and analyzed for antibody production using ELISA (~50 ng/well 6×HIS-hBFK was used to coat 96-well ELISA plates) assay as well as Western blotting. During this first screen, clones secreting antibodies were identified and subjected to expansion to 96-well format and a second screen was performed using the supernatants in ELISA and Western blotting tests.

2.8 Western blotting and *in situ* staining

Western blotting and *in situ* staining were carried out according to standard protocols. Antibodies were used in compliance with manufacturer's instructions. Specific conditions will be provided in detail upon request.

3 Results

3.1 Generation of highly specific rabbit and mouse anti-hBFK antibodies and hBFK protein expression patterns

Northern and dot blot analyses showed that the RNA for hBFK can be found specifically in the gastrointestinal tract [7] including the stomach, duodenum, jejunum, ileum, ilocecum, ascending, descending and transverse colon as well as the rectum. The two other tissues with clear expression of BFK are the pancreas and the lymph node (Özören N., data not shown). The pattern of expression for the human protein differs from that of the mouse homologue. BFK has been found to be expressed in the mouse mammary tissue, where its RNA and protein expressions were up-regulated after the induction of pregnancy, peaked at day 1 of lactation but then declined [6]. More recently, BFK expression in the mouse epididymal principal cells has been documented. In this tissue, RNA levels were influenced by androgens and other testicular factors and fluorescently tagged BFK was shown to be present in the nucleus [8].

We generated highly specific rabbit polyclonal sera and mouse mAbs against hBFK by injecting rabbits and mice with recombinant 6×HIS-tagged BFK, which was >95% pure after nickel column chromatography. We tested the specificity of the sera (rabbits 886 and 887) against tagged versions of BFK transfected into HEK293T cells. The antibodies recognize a single band at the expected size of 19 kDa, hBFK and the FLAG tag, which coincides with the protein detected using an mAb against the FLAG tag (Fig. 1A). We used the newly generated rabbit polyclonal sera to detect the endogenous protein in several human colon cancer cell lines such as SW480, HT-29 and HCT116, and found that SW480 and HCT116 cells express hBFK at high levels (Fig. 1A).

After verifying the specificity of the new IC10 hybridoma antibody by ELISA and Western blotting (data not shown), IC10 culture supernatant was used in a 1:2 dilution to detect the protein in human colon tissue by *in situ* staining (Fig. 1B), confirming the expression of hBFK in these tissues (Fig. 1B and data not shown). Isotype matching IgG control antibody was used as negative control. The specific gastrointestinal expression pattern makes hBFK a particularly interesting BCL-2 family member, suggesting a putative role in the unknown mechanism by which millions of cells are shed each day in the human gut.

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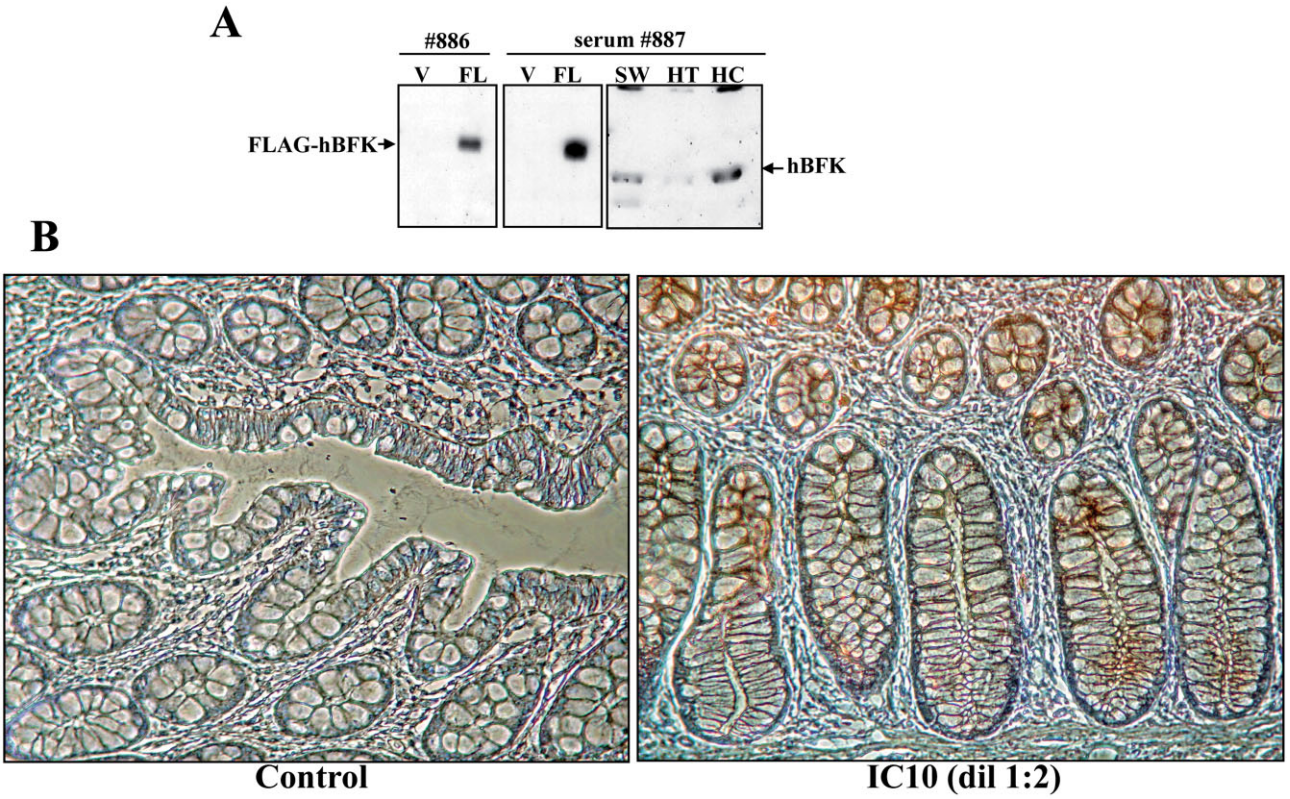

Figure 1. Highly specific anti-hBFK sera detect recombinant overexpressed and endogenous BFK protein. hBFK is expressed in the colon. (A) Lysates from HEK293T cells transfected with empty vector (V) (pcDNA3-FLAG) or FLAG-hBFK (pcDNA3-FLAG-hBFK) (FL) were tested for expression of recombinant tagged protein by Western blotting using polyclonal sera from rabbits 886 and 887, diluted 1:500. In the right panel serum 887 was used to detect endogenous protein in lysates from SW480 (SW), HT-29 (HT) and HCT116 (HC) colon cancer cell lines. The size of the endogenous protein is around 17.2 kDa, whereas the FLAG-tagged version is around 19 kDa. (B) Mouse anti-hBFK mAb-producing hybridoma supernatant (from clone IC10 at dilution 1:2) was used to detect BFK expression in the human colon. Isotype-matching control antibody was used as negative control for *in situ* staining.

3.2 hBFK interacts with BCL-XL, BCL-W but not BCL-2 or BAD

Different members of the BCL-2 family are known to interact with themselves as well as other family members. BH3 domain-only members are hypothesized to relieve the inhibition of the anti-apoptotic BCL-2 family members (BCL-2, BCL-XL and BCL-W) on the pro-apoptotic members such as BAX and BAK by disrupting their interactions [4, 5]. We found that hBFK interacts with the anti-apoptotic BCL-XL and BCL-W (Fig. 2A, lanes 4 and 8) but not with BCL-2 or BAD (Fig. 2A, lanes 6 and 10) in co-immunoprecipitation experiments carried out in 293T cells. The positive band in lane 3 shows the well-known interaction between BID and BCL-XL, which was used as a positive control for the assay. Lanes 2, 5, 7 and 9 contain the results of the negative control samples where only the corresponding FLAG-tagged protein was transfected together with empty vector pcDNA3-HA. Lack of co-immunoprecipitation cannot be attributed to the absence of protein expression, since adequate levels can be detected for all FLAG- tagged proteins (Fig. 2B) and HA-tagged hBFK (Fig. 2C). HA-tagged BID migrates differently from HA-hBFK and is not visible in the last panel.

These results further confirm the difference between the human and mouse proteins. In similar overexpression studies, it was found that mouse BFK does not interact with any BCL-2 family members [6].

3.3 BFK becomes activated through cleavage during DNA damage-induced apoptosis

To determine if hBFK has a pro-apoptotic function, the protein was overexpressed in HCT116 colon cancer cells. The full-length protein was not able to induce apoptosis, which suggested that it might need to be activated, as established for BID. We investigated whether hBFK becomes cleaved during doxorubicin-induced cell death, which is known to engage the signaling pathway of DNA damage-in-

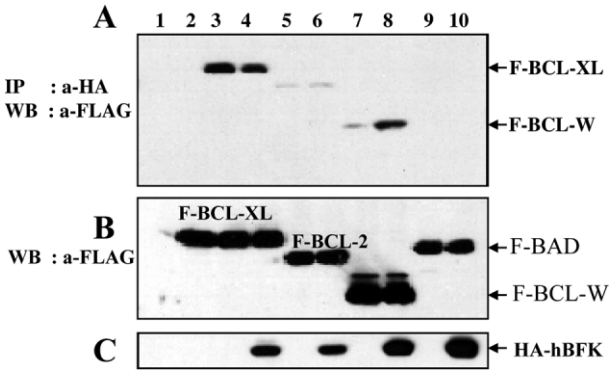

Figure 2. hBFK interacts with BCL-XL and BCL-W, but not with BCL-2 or BAD. (A) Co-immunoprecipitation of HEK293T cell lysates transfected with combinations of HA-tagged hBFK (HA-hBFK, lanes 4, 6, 8 and 10) and different FLAG-tagged BCL-2 family members (F-BCL-XL, lanes 2–4; F-BCL-2, lanes 5 and 6; F-BCL-W, lanes 7 and 8; F-BAD, lanes 9 and 10) was carried out using the anti-HA antibody, and precipitates were analyzed by Western blotting using the anti-FLAG antibody as described in Materials and methods. Lane 1 is a vector control negative lysate and lane 3 serves as a positive control, where HA-tagged BID was co-transfected together with F-BCL-XL. (B, C) Western blotting was carried out using the anti-FLAG and anti-HA antibodies on whole cell lysate samples (1/40) taken before the immunoprecipitation procedure to check the expression levels of the transfected proteins, as explained above.

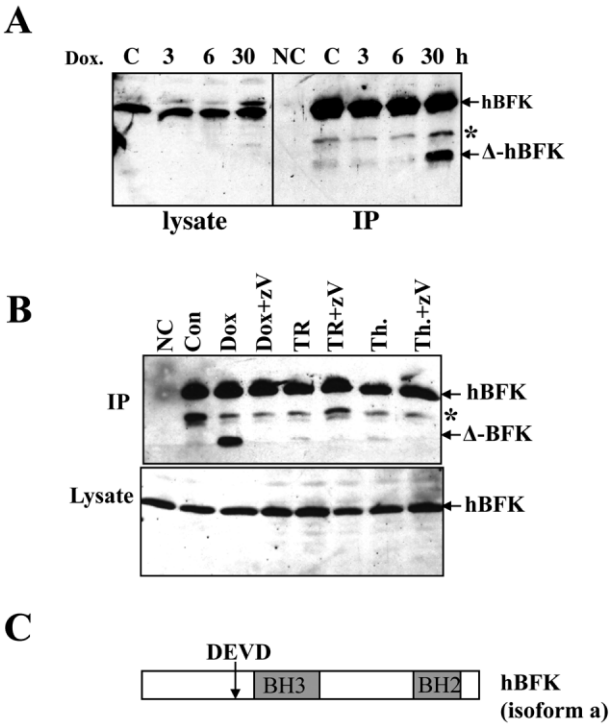

Figure 3. During DNA damage-induced apoptosis, endogenous hBFK becomes cleaved in a caspase-dependent manner. (A) Human colon cancer cells SW480 were treated with doxorubicin (0.5 μg/mL) for 3, 6 and 30 h and the presence of the cleaved hBFK band was visualized after immunoprecipitation using 10 μL anti-6xHIS-hBFK serum from rabbit 886 followed by Western blotting using the anti-6xHIS-hBFK mouse 3 serum at dilution 1/3000. NC, immunoprecipitation carried out with pre-bleed serum; and C, control doxorubicin-untreated cells. (B) SW480 cells were treated with doxorubicin (0.8 μg/mL), TRAIL (100 ng/mL) and thapsigargin (1 μg/mL) for 24 h in the presence or absence of the pan-caspase inhibitor zVAD-FMK (25 μM). In the upper panel, 10 μL serum from rabbit 886 was used to immunoprecipitate any endogenous BFK protein. Western blotting was performed using anti-6xHIS-hBFK serum of mouse 3 at dilution 1/5000. NC, immunoprecipitation carried out using a non-related anti-6xHIS antibody; Con, untreated cell lysates. In the lower panel the levels of hBFK in the lysates sampled before immunodepletion can be seen. (C) Diagram of isoform a of human BFK, showing the locations of the BH3 and BH2 domains as well as the location of the putative caspase 3, 6 or 7 cleavage site DEVD (not drawn to scale).

duced apoptosis. We treated SW480 cells with the drug for increasing lengths of time and observed the appearance of a cleaved form at 30 h post treatment (Fig. 3). These time points are consistent with the later onset of apoptosis under doxorubicin treatment, as previously reported. The detected cleaved form of hBFK, of ~14 kDa, corresponds with the expected protein after the utilization of the DEVD cleavage site at amino acid 38–41 of the human protein (Fig. 3C). At shorter incubation times (3 and 6 h), the cleavage product was present at negligible amounts comparable to untreated control samples (Fig. 4A). We were able to detect the endogenous cleavage product only after proteins were immunoprecipitated from solution using anti-hBFK polyclonal rabbit serum, followed by Western blotting with anti-hBFK mouse mAb hybridoma supernatant, produced for this purpose (Fig. 1).

After establishing the appearance of a cleaved band upon DNA damage induction, we wanted to test whether BFK also takes a role in death receptor-induced or ER stress-induced apoptosis in the SW480 colon cancer cells, and whether the cleavage was dependent on caspase activity (Fig. 3B). We treated cells with doxorubicin (DNA damage), TRAIL (death receptor signaling) or thapsigargin (ER stress) in the presence or absence of the pan caspase inhibitor zVAD (25 μM). We observed a clear cleavage product only in the doxorubicin-treated sample in the absence of the inhibitor (Fig. 3B). An intermediate shorter protein (denoted by *) is consistently detected in the immunoprecipitation samples but is unrelated to treatment, since it is present in the untreated controls. This band may represent caspase-independent but proteolytically digested protein or one of the shorter splice variants and needs further investigation.

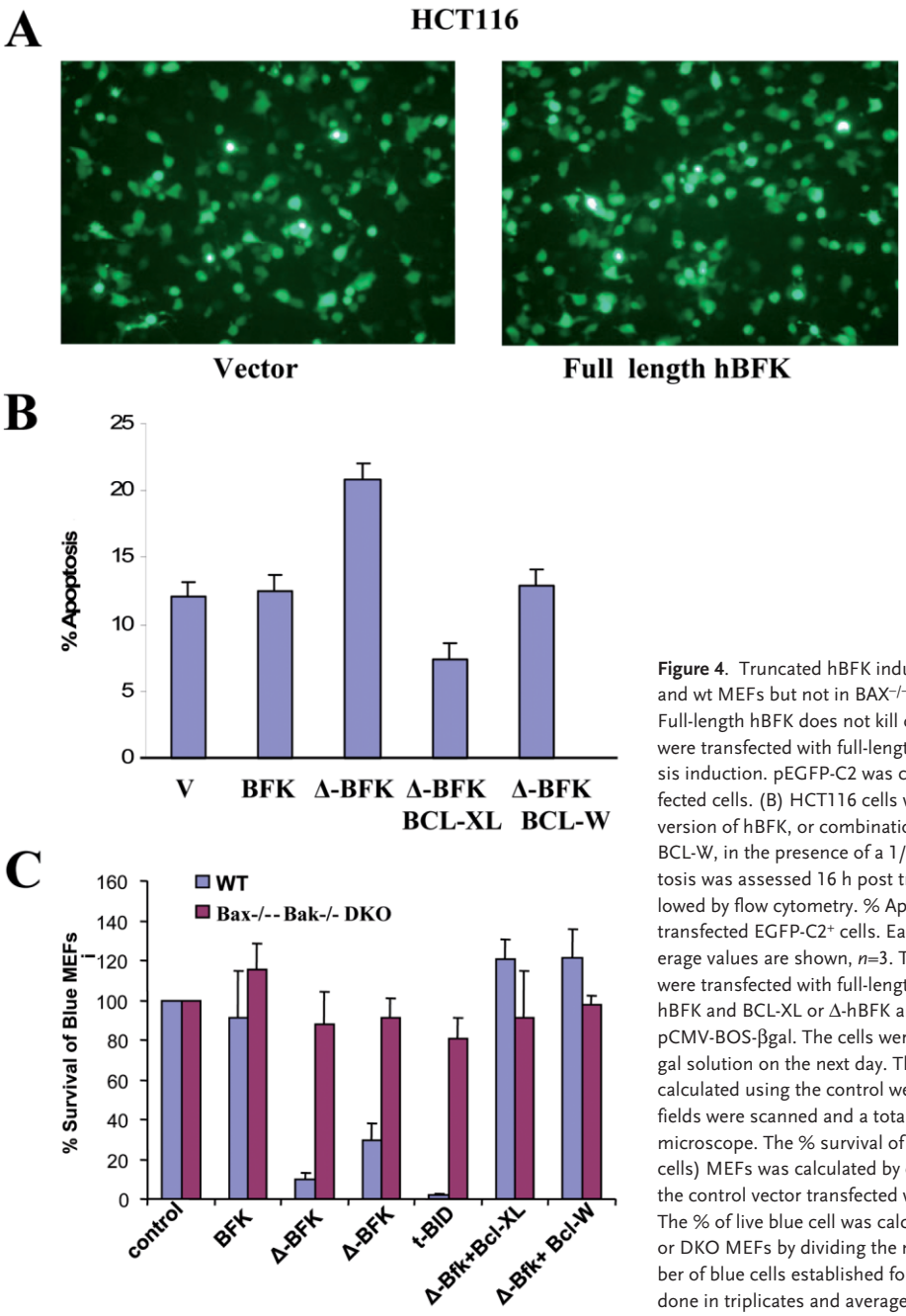

Figure 4. Truncated hBFK induces apoptosis in HCT116 colon cancer cells and wt MEFs but not in $BAX^{-/-}$-$BAK^{-/-}$ double-knockout (DKO) MEFs. (A) Full-length hBFK does not kill colon cancer cells HCT116. HCT116 cells were transfected with full-length hBFK or empty vector to analyze apoptosis induction. pEGFP-C2 was co-transfected at a 1/10 ratio to mark transfected cells. (B) HCT116 cells were transfected with full-length or truncated version of hBFK, or combinations of Δ-hBFK and BCL-XL or Δ-hBFK and BCL-W, in the presence of a 1/10 ratio of pEGFP-C2 vector DNA, and apoptosis was assessed 16 h post transfection by annexin V-PE staining followed by flow cytometry. % Apoptosis represents % annexinV-PE^+ / % transfected $EGFP\text{-}C2^+$ cells. Each condition was done in triplicates and average values are shown, n=3. The SEM is given. (C) DKO and wt MEFs were transfected with full-length or Δ-hBFK, tBID or combinations of Δ-hBFK and BCL-XL or Δ-hBFK and BCL-W, in the presence of a 1/10 ratio of pCMV-BOS-βgal. The cells were fixed after 24 h and stained with a 4% X-gal solution on the next day. The overall survival of transfected cells was calculated using the control wells, where for each well four to five visual fields were scanned and a total of 300 cells were counted using the light microscope. The % survival of blue (pCMV-BOS-βgal positive and live cells) MEFs was calculated by counting the number of total blue cells in the control vector transfected wt or DKO MEFs and setting that to 100%. The % of live blue cell was calculated for each condition separately for wt or DKO MEFs by dividing the number of viable blue cells by the total number of blue cells established for the control conditions. Each condition was done in triplicates and average values are shown. The SEM is given.

3.4 t-hBFK induces apoptosis in a BAX/BAK-dependent manner

To test whether the hBFK cleavage product or Δ-BFK has pro-apoptotic function, we transfected a plasmid encoding the hypothetical cleavage product, basically amino acids 42–163 of the human protein, into HCT116 colon cancer cells as well as wt and BAX-BAK double-knockout MEFs (Figs. 4B and C). In contrast to full-length hBFK protein, Δ-BFK induces apoptosis in the HCT116 cells and wt MEFs, which is inhibited by the simultaneous expression of BCL-XL or BCL-W (Figs. 4B and C). Apoptosis in HCT116 cells was twofold higher in the Δ-BFK transfected cells compared to the control and hBFK-transfected cells. hBFK-induced apoptosis was much more significant in the wt MEFs, where the rate of apoptosis was 70%. Truncated BID served as a positive control, since truncated mBID is known to require BAX or BAK for killing. In the Δ-hBFK transfected double-knockout MEFs the rate of apoptosis is much lower,

around 10%, suggesting that Δ-hBFK-mediated killing requires BAX or BAK.

4 Discussion

Apoptosis occurs in a precisely defined pattern in the gastrointestinal system. Stem cells are located at the crypts of Lieberkühn in the small intestine. These cells are positioned four to five cells up from Paneth cells at the base of the crypt. Cells migrate both upward to the villus tip to be shed, and to the crypt bottom to differentiate into Paneth cells. The rate of spontaneous apoptosis in the crypts is closely matched to the rate of proliferation, with 10% of the stem cells undergoing spontaneous apoptosis, which is independent of p53 [19, 20]. A large number of cells are lost at the tip of the villi *via* programmed cell death. In the large intestine, spontaneous apoptosis is not restricted to stem cells, which in this case are located at the crypt base, but rather occurs throughout the length of the crypt and a large number of senescent cells are shed at the plateau zones. BCL-2 family members are expected to have regulatory roles in the programmed cell death occurring in the intestine but relatively little is know about the mechanisms.

BFK is a newly identified member of the BCL-2 family with specific expression in the human gastrointestinal tract ([7] and N. Özören, unpublished data). In our study we focused on the human version of BFK and its possible physiological functions. We developed rabbit polyclonal sera and mouse mAb-producing hybridoma with high anti-hBFK specificities. We have shown that hBFK protein is expressed in the stomach and colon tissue and several colon cancer lines (Fig. 1 and data not shown). Furthermore, in co-immunoprecipitation experiments, hBFK clearly interacts with BCL-XL and BCL-W but not with BCL-2 or BAD (Fig. 2). We show for the first time that endogenous hBFK is cleaved in a caspase-dependent manner upon DNA damage induction in doxorubicin-treated SW480 colon cancer cells (Fig. 3). Finally, we provide completely novel evidence that a recombinant truncated protein, corresponding to the sequences downstream of the putative caspase 3, 6 or 7 cleavage site of the human protein, is capable of inducing apoptosis in HCT116 human cancer cells and in MEFs. Apoptosis induced by the Δ-BFK is clearly BAX and BAK dependent, since no killing is observed in their absence (Fig. 4).

The data presented here provide evidence that, like BID, hBFK also needs to be cleaved by a caspase to become a pro-apoptotic protein, suggesting that it acts as an amplifier of the apoptotic signal rather than an initiator. Full-length hBFK (isoform a) overexpressed in HEK293T cells or HCT116 colon cancer cells did not result in any apoptotic activity. Pro-apoptotic activity for isoform a was reported by Dempsey *et al*. [7] in A549 cells, and the discrepancy between our results may be attributed to the different cell lines that were used. Coultas *et al*. [6] have also observed a weak pro-apoptotic activity for the mouse BFK, structurally homologous to isoform a of the human protein, in HEK293T cells (10.5% compared to basal apoptosis levels of 6%). Based on the dramatic difference in the tissue distribution of the mouse and human orthologs of BFK, as well as the absence of the DEVD sequence in the mouse version, differences in function and regulation are expected. We show here that unlike the mouse BFK, hBFK does interact with several of the BCL-2 family proteins, the anti-apoptotic BCL-XL and BCL-W, but not BCL-2 or BAD. The ability to interact with BCL-XL and BCL-W but not BCL-2 is peculiar because they are all anti-apoptotic proteins; however, it is known for other pro-apoptotic BCL-2 family proteins that they do not display a general binding activity, but rather have preferred partners. A more detailed interaction study with the remaining BCL-2 family members as well as structure-based investigations will be necessary to define all the physiological partners of hBFK.

Another issue concerning the BFK orthologs is their cellular distribution [6–8]. Using our highly specific mouse anti-hBFK hybridoma supernatants, we have shown that the human BFK protein is localized to the cytoplasm (Fig. 1B and data not shown) of intestinal epithelial cells. However, the localization of the cleaved form remains to be documented.

BH3-only members NOXA [11, 12], PUMA [13] and BID [14] were shown to be regulated in a p53-dependent manner in response to DNA damage. We checked the upstream sequences of hBFK for p53 consensus binding sites and we found one. In preliminary experiments, we treated HCT116 colon cancer cells, carrying wt p53, with doxorubicin for increasing periods of time and clearly observed the stabilization of p53 protein, but no parallel increase in hBFK levels was present (N. Özören, unpublished data), suggesting that p53 does not transcriptionally regulate hBFK. Pujanto *et al*. [8] reported that mouse BFK levels are under the control of androgens and other testicular factors. The regulation of both mouse and human BFK proteins remains to be elucidated.

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The authors have declared no conflict of interest.

5 References

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Correction

Correction: Cloning and overexpression of a thermostable signal peptide peptidase (SppA) from *Thermoplasma volcanium* GSS1 in *E. coli*
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On page 1064, 5 lines from the bottom, it states "Lys is found at position 461 (*Ec* umbering) instead of Ala [28, 29]."

The correct sentence should be "However, in the *E.coli* enzyme, instead of Lys at position 461 (*Ec* numbering), there is Ala-461 [28, 29]."

We apologize with the authors and readers for any inconvenience.