Induction of Apoptosis by Human Nbk/Bik, a BH3-Containing Protein That Interacts with E1B 19K

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Received 15 March 1996/Returned for modification 12 June 1996/Accepted 20 June 1996

The E1B 19-kilodalton protein (19K protein) is a potent apoptosis inhibitor and the adenovirus homolog of Bcl-2 (E. White, Genes Dev. 10:1–15, 1996). To obtain a better understanding of the biochemical mechanism by which the E1B 19K protein regulates apoptosis, proteins that interact with 19K have been identified; one of these is Bax (J. Han, P. Sabbatini, D. Perez, L. Rao, D. Mohda, and E. White, Genes Dev. 10:461–477, 1996), and another is Bak (S. N. Farrow, J. H. M. White, I. Martinou, T. Raven, K.-T. Pun, C. J. Grinham, J.-C. Martinou, and R. Brown, Nature (London) 374:731–733, 1995). Bax and Bak are Bcl-2 family members which contain Bcl-2 homology regions 1, 2, and 3 (BH1, BH2, and BH3), which interact with E1B 19K and Bcl-2 and promote apoptosis. Like Bax and Bak, Nbk was cloned from a yeast two-hybrid screen for proteins that interact with E1B 19K. Nbk contained BH3 but not BH1 or BH2. It also interacted with Bcl-2 but not with Bax. Both Bcl-2 and E1B 19K interacted with Nbk in vitro, and this interaction was highly specific. In vivo, the Nbk and E1B 19K proteins may colocalize with cytoplasmic and nuclear membranes. Nbk expression functionally antagonized 19K-mediated inhibition of apoptotic cell death and completely prevented transformation by E1A and E1B 19K. Nbk was sufficient for induction of apoptosis in the presence of mutant p53 and thus low levels of Bax, suggesting that Nbk functions independently of Bax to induce apoptosis. Nbk may therefore represent a novel death regulator which contains only a BH3 that interacts with and antagonizes apoptosis inhibitors such as the E1B 19K protein.

It is widely recognized that apoptosis plays an important role in the normal development and differentiation of multicellular organisms and in numerous disease states (22). Appreciation of the importance of apoptosis to these processes has evoked considerable interest in the identification of gene products that function to regulate apoptosis (22). One advance in the understanding of apoptosis regulation was the discovery of the Bcl-2 family of apoptosis regulators. Even though Bcl-2 family members can either induce or inhibit apoptosis, they have common conserved regions, designated Bcl-2 homology regions 1, 2, and 3 (BH1, BH2, and BH3) (4, 5, 10). These regions are present in a wide variety of cellular and viral proteins. BH1, BH2, and BH3 are characterized by a small, conserved domain that was sufficient for the interaction of Bcl-2-related proteins suggested that other proteins that contained a BH3 domain may also be apoptosis regulators. Sequence analysis suggested the presence of a BH3 domain in one of the cDNAs that had previously been cloned in a two-hybrid screen with the E1B 19K protein as bait (10). This cDNA was originally described as Bp4 (10) and was later renamed Nbk or Bik. Bik is identical to Nbk and was isolated in a two-hybrid screen for Bcl-2-interacting proteins (2). Unlike other Bcl-2 family members, Nbk contained only BH3 and not BH1 or BH2. Nbk expression efficiently reversed the inhibition of apoptosis by the E1B 19K protein and was a potent stimulator of apoptotic cell death. Thus, apoptosis regulators such as Nbk that possess only a BH3 domain may represent a novel class of apoptosis regulators that may add another layer of complexity to the modulation of cell death by Bcl-2-related proteins.

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MATERIALS AND METHODS

Two-hybrid system. A HaLa cDNA library fused in the GAL4 activation domain of the pGAD-GH vector (11) was screened in Saccharomyces cerevisiae YH1 (ura3-25 his3-200 ade2-101 leu2-801 trp1-901 leu2-3C Can’ gal4-542 gal80-5 ade2-101 trp1-901 his3-157) as previously described (12). Briefly, the pGBT-9-19K plasmid was cotransfected with the pGAD library plasmid by standard lithium acetate transformation procedures. Transformants were plated on medium lacking Trp, Leu, and His. Approximately 3 × 10^6 transformants were screened for growth in the absence of histidine and assayed for β-galactosidase activity, as a second reporter, by a filter-based assay (11). The library plasmid was selectively recovered by transforming yeast miniprep DNA into Escherichia coli MH4 which is leuB + and therefore selects for the leucine marker on the pGBT-9 plasmid (11). Female-positive transformants were eliminated by screening for interaction with irrelevant proteins (Apac-2) and the empty pGBT-9 vector. Sequence analysis of the cDNA encoding the interacting proteins was performed with Sequenase 2.0 (U.S. Biochemical) as specified by the manufacturer.

Plasmid construction. Plasmids expressing E1B 19K, pm7, pm44, pm51, pm87, and pm102 in pGBT-9 have been described previously (10). Standard PCR were used to construct yeast fusion plasmids (hNbk in pGAD-GH, BH3-hnBk in pGAD-GH, and BH3-hnBk vector for in vitro transcription and translation of hNbk). The E1B 19K expression vector (pcDNA3-19K), the pT7 human Bcl-2 vector, the pT7-myc-hNbk vector for in vitro transcription and translation of hNbk (16). The pcDNA3-19K DNA was cotransfected with the pGAD library plasmid at the ratio 1:1, and the co-transformants were selected on YES selection media lacking histidine and leucine. The in vitro transcription and translation reactions were performed with a commercially available kit (Promega). The 32S-labeled proteins were separated by SDS-PAGE, visualised by autoradiography and quantitated using a phosphoimager (Molecular Dynamics).

RESULTS

Isolation of cDNA clones for E1B 19K-interacting proteins from a two-hybrid screen. The yeast two-hybrid system was used to identify novel cellular E1B 19K-binding proteins. We screened a HaLa (human) cDNA library constructed in the pGAD-GH vector, which contains the GAL4 activation domain, with the plasmid containing the E1B 19K coding region fused to the yeast GAL4 DNA-binding domain in the pGBT-9 vector (10). Three million transformants were screened by growth in the absence of histidine and for production of β-galactosidase by an X-Gal filter assay. Seven E1B 19K-interacting protein clones were sequenced, and one protein (Apac-2) was identified as a β-galactosidase-binding protein (10). Sequence data of these clones demonstrated that Apac-2 encoded part of human BAX (6). The BAX-binding protein was then incubated for 24 h to permit BAX protein expression. The cells were then fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) followed by 70% ethanol and incubated with biotinylated dUTP and TdT (0.3 U/ml; Boehringer Mannheim Biochemicals). Cells were washed with 0.125% bovine serum albumin in PBS, incubated with avidin-fluorescein isothiocyanate, and visualized by epifluorescence microscopy.

Nbk functional assays. To examine Nbk function in transient-expression assays, we used the pDAK3-19K construct was cotransfected with pCEP4-myc-hNbk into the E1A-plus-p53(Val-135) transformed cell line An1 by electroporation as previously described (3). The amount of transfected pDAK3-19K DNA was fixed at 10 μg and was cotransfected with either 1, 5, 10, or 20 μg of control vector or pCEP4-myc-hNbk DNA. The An1 cell line was also transfected with pCEP4-hnBk vector alone at the same concentration. The transfected cells were incubated at 38.5°C for 48 h and shifted to 32°C for 48 h. Viable-cell numbers were assessed by trypan blue exclusion.

A cotransfection assay with a β-galactosidase expression vector was also used to measure induction of cell death following Nbk transient expression. The An1 cell line was cotransfected with 6 μg of CMV-β-gal DNA expressing β-galactosidase on the cytomegalovirus promoter and 20 μg of control vector or pCEP4-myc-hNbk DNA. After 24 h of transfection, β-galactosidase activity was visualized by fixing cells with 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.4) 1 mM MgCl2, for 15 min and then washing them in PBS about five times. The fixed cells were stained for 4 h in PBS containing 3.3 mM each K3Fe(CN)6, H2O2, and K3Fe(CN)6, 150 mM NaCl, 1 mM MgCl2, and 0.2% 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal). The stained cells were observed microscopically, and the number of total cells and blue cells were determined.

For colony formation assays, the An1 cell line was transfected with 0.5, 1, or 5 μg of linearized pCEP4-myc-hNbk plasmid by electroporation. Stable cell lines were selected at 38.5°C and were screened by colony hybridization. Stable cell lines were selected at 38.5°C and were screened by colony hybridization. To infect cells with the ras oncogene, the cells were transfected with hNbk and the number of total cells and blue cells were determined.

In vitro protein interaction assays. mRNAs encoding human Bcl-2 (pGEM-2-Bcl-2), E1B 19K (pDNA3-19K), Bax (pDNA3-Bax), and hNbk (pDNA4-hNbk) were prepared from 6-day-old Fisher rats and were electroporated with test DNA plus carrier DNA. The primary rat cultures were cultured for 3 to 4 weeks at 38.5°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and the plates were then infected with the green fluorescent protein (GFP) plasmid in EX6 (15) with a diameter of 5 mm or larger were scored as positive for transformation. A minimum of eight plates for each plasmid combination were analyzed.

Isolation of cDNA clones for Bcl-2 proteins from a two-hybrid screen. The yeast two-hybrid system was used to identify novel cellular E1B 19K-binding proteins. We screened a HaLa (human) cDNA library constructed in the pGAD-GH vector, which contains the GAL4 activation domain, with the plasmid containing the E1B 19K coding region fused to the yeast GAL4 DNA-binding domain in the pGBT-9 vector (10). Three million transformants were screened by growth in the absence of histidine and for production of β-galactosidase by an X-Gal filter assay. Seven E1B 19K-interacting protein clones were sequenced, and one protein (Apac-2) was identified as a β-galactosidase-binding protein (10). Sequence data of these clones demonstrated that Apac-2 encoded part of human BAX including the BH3 region, which was sufficient to interact with the E1B 19K and Bcl-2 proteins in yeast strain YH1 (10). Bp4 (Nbk) encoded a 956-bp cDNA containing a 480-bp coding region in frame with GAL4 DNA-binding domain. Figure 1A shows the predicted amino-acid sequence of the coding region of Nbk.

To determine if there was similarity between Nbk and other sequences in the data banks, we used the FASTA, TFASTA (University of Wisconsin Genetics Computer Group package), and BLAST (1) algorithms. Nbk was not significantly similar to any other sequences in the data bases. However, a search for proteins that contained the BH3 interaction domain indicated that Nbk may be related to the BH3 region, which is sufficient to interact with the E1B 19K and Bcl-2 proteins in yeast strain YH1 (10). Bp4 (Nbk) encoded a 956-bp cDNA containing a 480-bp coding region in frame with GAL4 DNA-binding domain. Figure 1A shows the predicted amino-acid sequence of the coding region of Nbk.

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encode a membrane-spanning region, which is a characteristic feature of other Bcl-2 family members.

**Interaction of Nbk with Bcl-2 family members in yeast strain YGH1.** To test for interaction of Nbk with Bcl-2 family members other than the E1B 19K protein, the Nbk cDNA was cloned into the pGAD vector. Full-length human Nbk (hNbk) showed strong interaction with human Bcl-2 (hBcl-2) as well as with the E1B 19K protein (Fig. 2). The interaction of Nbk with Bcl-2 was also observed when Nbk was in the pGBT9 vector and Bcl-2 was in the pGAD vector (data not shown). Nbk, however, did not interact with rat Bax (rBax) or a nonspecific control protein (APC2) (Fig. 2). The specificity of Nbk for interaction with E1B 19K and Bcl-2, but not with Bax, was surprising since Bcl-2 and Bax are substantially homologous. This suggested that Nbk did not promiscuously interact with but, rather, distinguished between different members of the Bcl-2 family.

The BH3 region of Bax is sufficient for the interaction of Bax with either the E1B 19K protein or Bcl-2 (10). Nbk possesses a region with sequence homology to BH3, suggesting that Nbk may interact with Bcl-2 family members by the same means. To further characterize the specific binding region of Nbk required for interaction with either Bcl-2 or E1B 19K, we cloned the region encompassing the BH3 of Nbk (amino acids 48 to 76 [BH3-hNbk]) in the pGAD vector. BH3-hNbk was then examined for the ability to interact with E1B 19K and hBcl-2 in yeasts. BH3 of Nbk was sufficient for interaction with Bcl-2 but not with the E1B 19K protein (Fig. 2). The BH3 region of Nbk may therefore fulfill the same function as BH3 of Bax with respect to Bcl-2 interaction, but the binding requirements with the E1B 19K protein may be somewhat different. The equivalent region of Bcl-2 encompassing BH3 (amino acids 68 to 116 [BH3-hBcl-2]), was not capable of interaction with BH3 of Nbk (Fig. 2). This suggested the existence of nonsymmetrical interactions between Nbk and Bcl-2, which were also observed between Bax and E1B 19K (10).

**Sequence requirements within the E1B 19K protein for interaction with Nbk.** To analyze the specificity of the interaction between the E1B 19K and Nbk proteins, we used a panel of E1B 19K missense and deletion mutants. All five missense mutations (pm7, pm44, pm51, pm87, and pm102) produce a loss of E1B 19K function in vivo (4, 27). However, only pm51 (in BH3) and pm87 (in BH1) are completely defective for interaction with Bax in yeast strain YGH1 (pm51 is also defective for Bax interaction in vitro) (10). All of the E1B 19K deletion mutants are defective for Bax interaction, with the exception of the mutant with the deletion from 19 to 57 (encompassing BH3 of E1B 19K), which interacts with Bax weakly (10). E1B 19K mutants pm7, pm44, and pm102 sustained the ability to interact with Nbk (Fig. 3). Substitution of either phenylalanine for serine (pm51) within BH3 or glycine for alanine (pm87) within BH1 prevented or reduced the ability of
the E1B 19K protein to interact with Nbk (Fig. 3). On the basis of these missense mutations, the interaction requirements of the E1B 19K protein for Nbk and Bax appear to be the same. The missense mutation equivalent to pm87 in BH1 of Bcl-2 also inhibits both Bcl-2 function and Bcl-2–Bax interaction (28). BH1 is therefore likely to be important for protein conformation, interaction, and/or function of Bcl-2 and E1B 19K proteins.

The pm51 E1B 19K mutation, which is located in the BH3 region, failed to interact with Nbk (Fig. 3). pm51 also failed to interact with either BH3 or Bax (amino acids 50 to 78), or full-length rBax (10). Furthermore, the BH3 region of Bax is sufficient for the interaction with the E1B 19K protein in S. cerevisiae (10). This suggests that BH3, in addition to BH1, is important for E1B 19K protein interaction with Nbk and Bax and that this may affect E1B 19K function.

To further characterize the regions of the E1B 19K protein required for the interaction with Nbk, deletion mutants of the E1B 19K protein were analyzed. 19K deletion mutants contained amino-terminal (ΔN30, ΔN64, or ΔN87) or carboxy-terminal (ΔC46, ΔC93, or ΔC70) deletions or amino- and carboxy-terminal deletions (30 to 146, 30 to 93, 64 to 146, or 19 to 57) (10). Of all the deletion mutants, only the ΔC46 mutant interacted with Nbk (Fig. 3). Thus, although the BH1 and BH3 regions in the E1B 19K protein are important, the full-length E1B 19K protein, with the exception of the carboxy-terminal end, is required for interaction with Nbk.

Interaction of Nbk with E1B 19K protein in vitro. A GST-immobilized protein interaction assay was performed to confirm the ability of the GST-E1B 19K protein to bind to in vitro-translated Nbk protein. GST alone, GST-pm51 (E1B 19K BH3 missense mutant GST fusion protein defective for 19K-Nbk interaction in S. cerevisiae), and GST-E1B 19K fusion proteins were immobilized onto glutathione-Sepharose beads and purified (Fig. 4A). Equal amounts of GST-fusion proteins (3 μg) were mixed with [35S]methionine-labeled hBcl-2, rBax, or Nbk prepared by in vitro transcription and translation in buffer containing 0.2% NP-40. Production of in vitro-translated hBcl-2, rBax, and Nbk proteins was confirmed by immunoprecipitation with specific antibodies directed against hBcl-2 (ΔC-21), rBax (N-20), and the Myc epitope tag on Nbk (Myc antibody-1). A nonspecific E1B 55K antibody (13D2) was used as a negative control for nonspecific immunoprecipitation. In vitro-translated Nbk protein migrated with a molecular mass of 26 kDa, which was greater than the expected molecular mass of 18 kDa (Fig. 4B). The reason for this has not yet been determined.

As shown in Fig. 4B, in vitro-translated hBcl-2 did not bind to GST, GST-pm51, or GST-19K fusion proteins, as was observed previously (10). In vitro-translated rBax bound to only the GST-E1B 19K fusion protein and not to GST-pm51 or GST alone, as previously reported (10). Similarly, in vitro-translated Nbk bound only to GST-E1B 19K and not to GST-pm51 or GST alone (Fig. 4B). This assay was completely consistent with the finding of E1B 19K and Nbk interaction with the yeast two-hybrid system (Fig. 2) and provides independent evidence for an E1B 19K-Nbk interaction. Because of the insolubility of the E1B 19K protein in vivo, the use of standard coimmunoprecipitation assays to detect 19K-associated proteins has not been possible (23–25). As a consequence, dem-

FIG. 3. Sequence requirements within the E1B 19K protein for interaction with Nbk in yeast strain YGH1. Among the various serotypes, amino acids 1 to 81 ( ) share 52% identity, amino acids 82 to 113 ( ) is the most highly conserved region with 63% homology, and amino acids 114 to 176 ( ) is least conserved with 37% identity (4). The region required for transformation and inhibition of apoptosis (amino acids 44 to 113) is indicated (4, 27). BH1, BH2, and BH3 are indicated as I, II, and III, respectively. The interactions were measured by growth on histidine-deficient plates and by X-Gal staining. Symbols: ++++, strong interaction; +, weak interaction; −, no interaction. E1B 19K missense and deletion mutations have been described previously (4, 10, 27).
onstration of a direct interaction between 19K and Nbk (or any other protein) in vivo has been difficult.

Evidence for colocalization of Nbk and E1B 19K in vivo. To determine if Nbk and E1B 19K proteins colocalize in the same subcellular compartment, indirect immunofluorescence was performed. Since transient Nbk expression in BRK cells induced cell death (see below), we transfected a Myc-tagged Nbk mammalian expression vector (pCEP4-myc-hNbk) into the 19K1 cell line. The 19K1 cell line is an E1A-plus-tsp53(Val-135)-transformed BRK cell line that stably expresses the E1B 19K protein and is thereby substantially resistant to apoptosis (6). 19K1 cells were transfected with the Nbk expression vector, and at 48 h posttransfection, the cells were fixed and double-stained with a mouse monoclonal antibody specific for the Myc epitope on Nbk and a polyclonal antibody directed against the E1B 19K protein. Nbk and the E1B 19K proteins were visualized with rhodamine- and fluorescein-conjugated anti-mouse and anti-rabbit secondary antibodies, respectively.

As previously reported (23–25), the E1B 19K protein was detected exclusively in cytoplasmic and nuclear membranes (Fig. 5A). Nbk appeared to colocalize with the E1B 19K protein at the nuclear envelope and cytoplasmic membranes (Fig. 5B). More than 100 Nbk-expressing cells were examined, all of which displayed a localization pattern indistinguishable from the representative photograph shown in Fig. 5. E1B 19K and Nbk may be expected to reside in the same intracellular compartment.

Nbk induces cell death and antagonizes E1B 19K protein function. To determine the functional relationship between Nbk and the E1B 19K proteins with respect to the regulation of apoptosis, the Nbk expression vector (pCEP4-myc-hNbk) and/or the E1B 19K expression vector (pcDNA3-E1B 19K) was transfected into the An1 cell line. An1 is a BRK cell line transformed by E1A and tsp53(Val-135) (6). At the restrictive temperature for p53 (38.5°C), An1 cells are transformed and proliferate, but at the permissive temperature (32°C), they undergo p53-dependent apoptosis (3, 6, 10, 13, 17, 18). Transient expression of E1B 19K protein in An1 cells, however, blocks the induction of p53-mediated apoptosis at the permissive temperature and thereby provides an efficient assay for E1B 19K protein function (10).

Transient E1B 19K expression increased the viability of the An1 cell line by approximately fivefold over the vector alone at the permissive temperature (Fig. 6). Transfection of the An1 cell line with increasing concentrations of the Nbk expression vector reduced viability even further than did transfection with the control vector, which suggested that Nbk expression promoted apoptosis (Fig. 6). Cotransfection of increasing concentrations of Nbk with the E1B 19K expression vector completely

FIG. 4. Interaction of the E1B 19K protein with Nbk in vitro. (A) Production of GST-fusion proteins. GST, GST-pm51, and GST-E1B 19K fusion proteins produced in BL21 were purified on glutathione-Sepharose beads. The purity and quantity of GST fusion proteins were estimated by SDS-PAGE. (B) Association of the GST-E1B 19K protein with in vitro-translated hNbk. The GST, GST-pm51, and GST-E1B 19K fusion proteins were incubated with in vitro-translated hBcl-2, rBax, and hNbk in immunoprecipitation buffer. The in vitro-translated hBcl-2, rBax, and hNbk were recovered on glutathione beads and analyzed by SDS-PAGE. Immunoprecipitations of in vitro-translated hBcl-2 (Δ-C21 antibody), rBax (N-20 antibody), and hNbk (Myc antibody 1) in the binding assay are shown. The 13D2 antibody directed against the E1B 55K protein was used as a control for nonspecific immunoprecipitation.

FIG. 5. Intracellular localization of E1B 19K and Nbk proteins in BRK cells. The 19K1 cell line was transfected with Nbk expression vector (pCEP4-myc-hNbk) or control vector (pCEP4) at 38.5°C and incubated for 48 h at 32°C. The transfected cells were stained with an E1B 19K polyclonal antibody (p21) or Myc antibody to detect the amino-terminal epitope tag on the Nbk protein 24 h posttransfection. Fluorescein-conjugated secondary antibody was used to visualize the E1B 19K protein (A), and rhodamine-conjugated secondary antibody was used to visualize Myc (Nbk) (B). Staining of E1B 19K (A) and Nbk (B) in the same field of cells is shown. Bar, 100 μm.
abrogated the ability of the E1B 19K protein to inhibit apoptosis, reducing viability to that observed when no E1B 19K protein was expressed (Fig. 6). The reduction of viability by Nbk was proportional to the amount of transfected Nbk DNA (Fig. 6). Thus, transient expression of Nbk induced cell death in the An1 cell line and significantly and dramatically repressed the antiapoptotic function of the E1B 19K protein.

**Induction of apoptosis by Nbk occurs downstream of p53.**

The loss of viability upon Nbk expression in Fig. 6 suggested that Nbk may stimulate apoptotic cell death. To determine if the cell death induced by transient Nbk expression was caused by apoptosis, a TdT assay (8) was used to detect the fragmentation of DNA in nuclei, which is one of the typical events associated with apoptosis.

19K1 cells were transfected with the Nbk expression vector or a vector control and incubated at 38.5°C for 24 h. In the case of cells transfected by the vector control, only very few TdT-positive cells were detected (Fig. 7A, panel 1). However, cells transfected with the Nbk expression vector showed a greater than fourfold increase in the number of TdT-positive cells, which, in addition, displayed nuclear fragmentation and cytoplasmic morphological changes associated with apoptosis (Fig. 7A, panel 2). These results indicated that induction of cell death by Nbk expression is caused by a process that resembles apoptosis.

To address whether the induction of apoptosis by Nbk required a functional p53 protein, we performed β-galactosidase assay and colony formation assay in An1 cells at the restrictive temperature, at which tsp53 is primarily in the mutant conformation (6). For the β-galactosidase assay, the An1 cell line was transfected with the Nbk expression vector or control vector and incubated at 38.5°C for 24 h. The cells were fixed with 1% glutaraldehyde and stained in PBS containing X-Gal. The stained cells were observed microscopically, and the percentage of blue cells was determined.

**FIG. 6.** Nbk induces cell death and inhibits the antiapoptotic function of the E1B 19K protein. Increasing quantities (1, 5, 10, and 20 μg) of the hNbk expression vector DNA (pCEP4-myc-hNbk) or control vector DNA (pCEP4) were cotransfected into the An1 cell line with a fixed amount (10 μg) of E1B19K expression vector DNA (pcDNA3-E1B 19K) or control vector DNA (pcDNA3). The cotransfected An1 cell lines were incubated at 38.5°C for 48 h and then at 32°C for 48 h. Viable-cell numbers were measured by trypan blue exclusion.

**FIG. 7.** Induction of apoptosis by Nbk does not require a functional p53. (A) Induction of DNA fragmentation by transient Nbk expression. The TdT assay was used to detect DNA fragmentation in situ. The 19K1 cell line was transfected with the Nbk expression vector (pCEP4-myc-hNbk) or the control vector (pCEP4), incubated for 24 h at 38.5°C, and then processed for the TdT assay as described in Materials and Methods. Magnification, ×600. (B) Induction of cell death by transient Nbk expression in the An1 cell line. The An1 cell line was cotransfected with pCMV–β-Gal DNA and control vector DNA or pCEP4-myc-hNbk DNA at a 1:3 ratio and incubated at 38.5°C for 24 h. The cells were fixed with 1% glutaraldehyde and stained in PBS containing X-Gal. The stained cells were observed microscopically, and the percentage of blue cells was determined. (C) Inhibition of colony formation by Nbk expression. The An1 cell line was transfected with increasing concentrations (0.5, 1, 2, and 5 μg) of linearized Nbk expression vector DNA (pCEP4-myc-hNbk) or control vector DNA (pCEP4) and incubated at 38.5°C for 21 days in medium containing hygromycin. The number of colonies was assessed by Giemsa staining.
cotransfected with CMV-β-gal DNA and pCEP4-myc-hNbk DNA at a 1:3 ratio respectively and incubated at the restrictive temperature. At 24 h after transfection, β-galactosidase activity was detected histochemically. Transfection of vector control DNA resulted in approximately 22% blue cells, whereas transfection with either Bax or Nbk resulted in 1 to 2% blue cells (Fig. 7B). Thus, Nbk expression was incompatible with cell viability and the level of biological activity of Nbk was comparable to that of Bax. In a colony formation assay, the Nbk expression vector was transfected into the An1 cell line at the restrictive temperature. An1 cells transfected with either Nbk or the control vector were incubated at the restrictive temperature in medium containing hygromycin to select for stable transformants that express Nbk. At 21 days posttransfection, the number of hygromycin-resistant colonies was determined by Giemsa staining. The number of colonies in the Nbk transfec-tants was decreased by more than fourfold compared with the control vector (Fig. 7C). This result is typical of enforced expression of a toxic gene product, such as Bax, which stimulates apoptosis and is therefore incompatible with cell viability and colony formation (10). This also suggested that Nbk expression was sufficient for the induction of apoptosis and did not require coexpression of any cofactors in BRK cell lines to implement the apoptotic response.

We have previously shown that bax is transcriptionally induced by p53 in the BRK cell lines and that bax expression is sufficient for the induction of apoptosis, suggesting that it acts downstream of p53 (10). At the restrictive temperature for p53, at which the experiment in Fig. 7 was performed, Bax levels are exceedingly low, owing to the absence of wild-type p53 (10). Since Nbk expression apparently induced apoptosis in the virtual absence of Bax, Nbk function did not require Bax and may be redundant to Bax. In addition, Northern (RNA) blot analysis revealed that nbk is constitutively expressed at both the restrictive and permissive temperatures and, unlike bax, is not transcriptionally upregulated by p53 (data not shown). Taken together, these results indicate that induction of apoptosis by Nbk probably occurs independently of Bax and downstream of functional p53.

Nbk inhibits transformation by E1A and E1B 19K. To further assess the functional relationship between Nbk and the E1B 19K protein, we determined if Nbk could impair the ability of the E1B 19K protein to cooperate with E1A in the transformation of BRK cells. Transfection of E1A alone produced very few foci because of the induction of apoptosis (16, 26, 27), and cotransfection of E1A with either Bax or Nbk resulted in even fewer foci (Fig. 8). This result is consistent with the fact that both Bax and Nbk are potent inducers of apoptosis in BRK cells (10) (Fig. 7). Cotransfection of E1B 19K with E1A resulted in an average of 15 foci per plate, whereas cotransfection of Bax with E1A and E1B 19K resulted in an average of 7 foci per plate (Fig. 8). This ability of Bax to compromise the transforming potential of the E1B 19K protein is consistent with the fact that Bax binds to the E1B 19K protein and antagonizes its antiapoptotic activity (10). Nbk, however, abrogated transformation entirely, suggesting that it is a more potent activator of apoptosis than Bax (Fig. 8).

DISCUSSION

We have identified a new type of apoptosis regulator, Nbk, that is related to the Bcl-2 family only because it possesses a BH3. Nbk interacts with both the E1B 19K and Bcl-2 proteins, which was expected since the 29-amino-acid region surrounding BH3 of Bax is sufficient for E1B 19K interaction in S. cerevisiae (10). Similarly, a 45-amino-acid region surrounding BH3 of Bak is sufficient for Bcl-x, or E1B 19K interaction (5). BH3 probably represents an important protein interaction domain and may confer the ability to bind to Bcl-2-related proteins and regulate apoptosis. Nbk (also known as Bik) was independently cloned by two other groups, also by using a two-hybrid screen for Bcl-2- and E1B 19K-interacting proteins (2, 15). In these studies, Nbk expression either stimulated apoptosis on its own, as we have found, or sensitized cells to the induction of apoptosis by Fas antigen. Whether Nbk is one member of a multigene family or is unique remains to be determined.

Previous evidence suggests that Bcl-2 and Bax regulate cell death by a “dueling-dimer” or “rheostat” model, in which the ratio of Bcl-2, which inhibits cell death, to that of Bax, which promotes cell death, controls cell viability (14). Definitive evidence whether Bcl-2 or Bax is the effector molecule is so far lacking. The identification of Nbk adds another layer of complexity to this situation and suggests that a dueling-dimer or rheostat model may not be sufficient to account for apoptosis regulation.

How could Nbk contribute to apoptosis? One possibility is that any protein containing BH3 may induce apoptosis merely by sequestering Bcl-2-like apoptosis inhibitors. In that case, Bcl-2 would be the effector that is inhibited by interaction with a BH3-containing protein such as Bax or Nbk. In support of this model, both Bax and Nbk efficiently kill cells, yet all they have in common is BH3.

Conversely, BH3 in the context of Bax, Bak, and Nbk may be a death domain. In support of this scenario, deletion mutant analysis of Bak has demonstrated that loss of BH3 prevents the lethality of Bak and Bik (5). More significantly, however, expression of the region of Bak slightly larger that BH3 is sufficient to induce apoptosis (5). Since Bcl-2 also possesses BH3 but does not induce cell death, any protein that contains BH3
does not confer lethality when expressed. Perhaps the context of BH3 may determine whether it has death-promoting activity. An alternative possibility is that Nbk is a death effector. Nbk binds only to Bcl-2 and E1B 19K, not to Bax. Furthermore, unlike Bax, Nbk is apparently not a p53-inducible gene (10). Complexes between Bcl-2 (or E1B 19K) and Nbk may preexist in cells. In situations when p53 function is activated, such as with E1A expression in BRK cells, for example, Bax transcription is upregulated by p53 (10). Bax, which contains BH1 to BH3, is more homologous to Bcl-2 than to Nbk and may displace Nbk by forming tighter complexes with Bcl-2. Newly liberated Nbk would then implement apoptosis. Mutational analysis of Nbk should be useful in establishing which one of these possible models for the regulation of apoptosis is correct. In functional assays, Nbk is a potent inhibitor of E1B 19K function and can completely abrogate transformation by E1A and E1B 19K. This raises the possibility that Nbk is a tumor suppressor gene product. This and any potential role for Nbk in the regulation of apoptosis in development remain to be determined.

ACKNOWLEDGMENTS

We thank Linda Van Aelst and Maria Fernandez-Sarabia for providing the HeLa cDNA library in pGAD-GH and Céline Gélinas for human Bcl-2 in pGEM-2. We also thank D. Perez, L. Rao, S. Chiou, K. Degenhardt, and G. Kasof for helpful comments and suggestions. This work was supported by grants from NIH (CA 53370 and CA 64807) to E.W.

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