MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3884–3892 Vol. 16, No. 7
0270-7306/96/$04.00+0

The p53-Binding Protein 53BP2 Also Interacts with Bcl2 and Impedes Cell Cycle Progression at G_2/M

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Received 17 October 1995/Returned for modification 20 November 1995/Accepted 25 March 1996

Using the yeast two-hybrid system, we have isolated a cDNA (designated BBP, for Bcl2-binding protein) for a protein (Bbp) that interacts with Bcl2. Bbp is identical to 53BP2, a partial clone of which was previously isolated in a two-hybrid screen for proteins that interact with p53. In this study, we show that specific interactions of Bbp/53BP2 with either Bcl2 or p53 require its ankyrin repeats and SH3 domain. These interactions can be reproduced in vitro with bacterially expressed fusion proteins, and competition experiments indicate that Bcl2 prevents p53 from binding to Bbp/53BP2. BBP/53BP2 mRNA is abundant in most cell lines examined, but the protein cannot be stably expressed in a variety of cell types by transfection. In transiently transfected cells, Bbp partially colocalizes with Bcl2 in the cytoplasm and results in an increased number of cells at G_2/M, possibly accounting for the inability to obtain stable transfectants expressing the protein. These results demonstrate that a single protein can interact with either Bcl2 or p53 both in yeast cells and in vitro. The in vivo significance of these interactions and their potential consequences for cell cycle progression and cell death remain to be determined.

Apoptosis, or programmed cell death, is a generally recognized process that occurs under a variety of conditions ranging from development to chemotherapy-induced cell death. Cells undergoing apoptosis shrink, develop condensed chromatin, degrade their DNA into oligonucleosomal fragments, and then lose viability.

The genes that have been implicated as being involved in apoptotic pathways include *BCL2*, which inhibits apoptosis, and the p53 gene, which can initiate growth arrest and apoptosis. The *BCL2* oncogene was initially identified at the breakpoint of t(14;18)(q32;q21), the most common chromosomal translocation in human lymphoid malignancies (3, 10, 50). This translocation juxtaposes the immunoglobulin heavy-chain gene enhancer with the *BCL2* gene, resulting in constitutive expression of *BCL2* transcripts with subsequent overexpression of the 24- to 26-kDa Bcl2 protein (7, 11). Overexpression of Bcl2 in lymphoid cells in culture and transgenic mice prevents apoptosis induced by a wide variety of agents such as steroids, gamma irradiation, and growth factor deprivation (21, 28, 34, 41, 45, 47, 52). Some of the functions of Bcl2 may be mediated through interaction with Bax, a Bcl2 homolog (35, 57). Bcl2 also protects against differentiation-associated cell death (33, 36). Loss of *BCL2* in knockout mice does not interfere with normal development of T and B cells, but fulminant apoptosis of T and B cells occurs after several weeks of age (32, 53). Additionally, the mice are born with kidney abnormalities and develop gray hair after 1 month of age. These and other studies have implicated a role for Bcl2 in a reactive oxygen species pathway; however, fulminant apoptosis of lymphoid cells in mice may be due to other factors such as a surge in glucocorticoid hormones during mouse maturation (22, 55).

Apoptosis is also modulated by intracellular levels of p53. p53 was identified as a cellular protein bound by simian virus 40 large T antigen and was initially believed to be an oncogene; however, later work showed that its role is more consistent with that of a tumor suppressor (20). p53 is required for G_1 arrest of cells after treatment with DNA-damaging agents and therefore is a cell cycle checkpoint protein (25). p53 is mutated in up to 80% of a variety of different tumors, and although it is not an essential gene for mouse development, its absence leads to an increased incidence of spontaneous tumors (13). Wild-type p53 can induce apoptosis when reintroduced into cells which lack it (37, 42, 59). Wild-type p53 is also required for radiation-induced apoptosis but not for glucocorticoid-induced apoptosis in mouse thymocytes (27). The cell cycle arrest but not apoptosis effected by p53 may be explained by its ability to induce transcription of various genes such as *WAF1/CIP1*, which codes for an inhibitor of cyclin-dependent kinase, thereby leading to growth arrest (15, 19). Apoptosis induced by p53 is blocked or delayed by overexpressed Bcl2, but cells continue to be growth inhibited, further suggesting that these functions of p53 are separable and that Bcl2 affects only the p53-induced apoptosis pathway (29, 46). This antagonistic relationship between Bcl2 and p53 is not well understood. It has been shown that p53 down-regulates Bcl2 expression and up-regulates Bax expression in the murine leukemia cell line M1, thereby leading to apoptosis (40).

The studies reported here were undertaken to identify proteins that interact with Bcl2. Using the yeast two-hybrid system, we have isolated a cDNA that encodes a protein which interacts with Bcl2 (14, 18). Bbp is unrelated to Bcl2 family members or other proteins known to interact with Bcl2, including Bcl2 itself, R-Ras p23, Bax, Bcl-x_L, Bcl-x_S, Mcl-1, Nip1, Nip2, Nip3, Bak, and Bad (5, 6, 8, 16, 17, 26, 35, 39, 56–58). Unexpectedly, Bbp also interacts with p53 and is identical to 53BP2, a protein previously isolated in a two-hybrid screen for proteins that interact with p53 (24). In this study, we show that Bbp ankyrin repeats and SH3 (Src homology 3) domain are required for interaction with Bcl2 or p53 in the two-hybrid system and that these interactions can be reproduced in vitro with bacterially expressed fusion proteins. We also show that Bbp/53BP2 is a cytoplasmic protein that partially colocalizes

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with Bcl2. Bbp/53BP2 cannot be stably expressed but upon transient expression appears to perturb the cell cycle at G_2/M.

MATERIALS AND METHODS

Strains and plasmids. Yeast strains, plasmids, and the cDNA library used for yeast two-hybrid screening have been previously described (14). The *BCL2* cDNA from a synthetic *Eco*RI site to an internal *Bam*HI site was cloned into the pAS1 vector to generate a fusion protein consisting of the Gal4 DNA binding domain (DBD) fused to Bcl2 (11). The fusion protein encoded by the bait plasmid contained a 12-amino-acid spacer between the Gal4 DBD and Bcl2 followed by the first 188 amino acids of Bcl2. The 51 carboxy-terminal amino acids of Bcl2 containing the membrane insertion domain were not encoded by the construct in an effort to minimize the possibility of the bait Bcl2 protein inserting into cytosolic membranes (endoplasmic reticulum, plasma membrane, and mitochondria) and not being targeted to the nucleus through the *GAL4* nuclear localization domain. The bait plasmid was transformed into yeast strain Y153, and Western blotting (immunoblotting) was performed with an anti-Bcl2 monoclonal antibody to verify that fusion protein of the expected molecular mass (approximately 39 kDa) was produced (data not shown). Cell fractionation studies demonstrated that the protein was primarily found in the nuclear fraction and not in the cytoplasmic or mitochondrial fractions (data not shown).

Isolation of library plasmids coding for Bcl2-interacting proteins. Yeast strain Y153 expressing the DBD-Bcl2 fusion protein was unable to grow on minimal medium with 3-amino-1,2,4-triazole (3-AT) concentrations greater than 25 mM and did not produce β-galactosidase. Strain Y153/DBD-BCL2 was transformed with a human cDNA library (prepared from Epstein-Barr virus-transformed human peripheral blood lymphocytes) fused to the Gal4 activation domain (AD) in the pACT vector (14). Transformed yeast cells were plated on minimal medium containing 50 mM 3-AT. After 7 to 10 days, numerous small colonies were noted; however, there was no outgrowth of large colonies as we have noted in other two-hybrid screens, possibly because of overcrowding of transformants. These plates, representing approximately 1 million transformants, were separated into four different pools, and the cells were scraped off and grown in minimal medium with 50 mM 3-AT for 3 days. The cells were then replated on 50 mM 3-AT. After 1 week, two pools yielded large colonies capable of growth on 50 mM 3-AT. These colonies also tested positive for in situ β-galactosidase expression on 3-AT plates. Curing of either the bait or library plasmid resulted in loss of resistance. Single library plasmids (pAD-BBP^b and pAD-BBP^c) capable of transferring 3-AT resistance to Y153/DBD-BCL2 were isolated from each strain and used for further studies which showed that they originated from the same gene, designated BBP. Both of these plasmids were proficient at transferring 3-AT resistance and β-galactosidase activity when cotransformed into Y153 (data not shown).

In vitro protein interactions. A plasmid expressing a glutathione *S*-transferase (GST)–53BP2 fusion protein (referred to in this report as GST-Bbp) was obtained from Bin Li and Stan Fields (State University at Stony Brook) and has been described previously (24). Bcl2 and p53 fusions to maltose-binding protein (MBP) were constructed in the Malc2 vector. A strain producing control protein MBP-paramyosin was obtained from Paul Riggs (New England Biolabs, Beverly, Mass.). Bacteria containing fusion plasmids (100 ml for MBP fusions and 500 ml for GST-Bbp fusion) were grown at 30°C, induced with isopropylthiogalactopyranoside (IPTG), and sonicated as described previously (43) except that the buffer consisted of 20 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM dithiothreitol (DTT) (buffer A). Extracts were used directly or frozen in dry ice-ethanol and stored at −70°C. For in vitro binding, 10 μl of GST fusion protein (approximately 10 μg) and 100 μl of MBP fusion (approximately 100 μg) were mixed and brought up to 200 μl with lysis buffer. DTT was added to 5 mM, and the tubes were incubated at 35°C for 1 h. Glutathione-agarose (total of 100 μl; 25-μl gel volume in lysis buffer with 1 mM DTT) was added, and the tubes were rocked gently for 15 min at room temperature. The beads were washed five times with buffer B (20 mM Tris [pH 7.5], 300 mM NaCl, 0.5% Triton X-100, 1 mM DTT). Protein loading buffer (30 μl) was added, and 15-μl samples were loaded on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Protein binding was monitored by staining with Coomassie blue and Western blotting with an anti-MBP antibody (1/10,000; New England Biolabs) followed by horseradish peroxidase anti-rabbit immunoglobulin G (1/10,000) and were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

For analysis of competitive binding, a crude extract containing approximately 1 μg of GST-Bbp was added to crude extracts containing 100 μg of either MBP-Bcl2 or MBP-p53, and the mixture was incubated to allow complex formation. Various amounts of the other competitor protein were then added, and incubation continued for 1 h. Complexes were analyzed as described above.

Immunoprecipitations of in vitro protein complexes. Extracts containing GST-Bbp, MBP-Bcl2, and MBP-p53 were mixed, incubated to form complexes, and split into separate tubes. Mouse anti-GST or anti-Bcl2 antibodies were added (1/100), and the mixtures were incubated on ice for 1 h. To enhance immunoprecipitation, rabbit anti-mouse antibody (0.5 μl) was added for 30 min, then protein A-Sepharose was added, and the mixture was subjected to washing with buffer B and Western blot analysis with anti-MBP antiserum. In a complementary experiment, GST-Bbp/MBP fusion protein complexes were purified by glutathione resin affinity chromatography prior to immunoprecipitation and Western blot analysis.

Isolation and characterization of full-length BBP cDNAs. The BBP cDNA fragment isolated from yeast two-hybrid screening was used as a hybridization probe to screen a cDNA library constructed from the B-cell progenitor cell line HAL-01 (23). The two longest cDNAs, approximately 4.5 kb in length, were sequenced, one in its entirety on both strands and the other partially. The clones differed slightly at their 5′ and 3′ ends, and each contained all of the sequences present in pAD-BBP^c. Both contained an *Alu* repeat element in their 5′ untranslated regions. To define the actual protein translation start site, in vitro coupled transcription-translation was performed with cDNAs containing progressively larger deletions at their 5′ ends. This localized the initiating ATG between the *Bam*HI and *Sac*I sites in the cDNA at nucleotide 757, which would result in a predicted protein of 1,005 amino acids.

Epitope tagging of BBP and mammalian cell transfections. A potential initiating ATG codon was defined on the basis of in vitro translation of various deletion BBP constructs. Since this ATG is preceded by a natural *Avr*I site, an *Avr*I-*Xba*I fragment of BBP was cloned into an SRα promoter (48) expression vector designed to place a FLAG epitope tag at the 5′ end of the open reading frame (PyDF30; constructed by D. Forentino, Stanford University). COS cells were transfected by a standard calcium phosphate precipitation technique, and proteins were isolated by extraction of whole cells in SDS-containing buffer. Western blotting was performed by standard techniques, using anti-FLAG antibodies at a dilution of 1/1,000 or rabbit anti-BBP antibody at a dilution of 1/500. Secondary antibody, horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G, was used at a dilution of 1/5,000, and visualization was by enhanced chemiluminescence (Amersham). For long-term transfections with selection, FLAG epitope-tagged BBP and NFAT were cloned downstream of the cytomegalovirus promoter in CEP4 (CEP-FBBP and CEP-FNFAT, respectively; Invitrogen, San Diego, Calif.). CEP4 does not contain a simian virus 40 origin of replication (ori) but can replicate episomally in mammalian cells because it expresses Epstein-Barr virus nuclear antigen 1, oriP, and a selectable marker encoding hygromycin resistance. Constructs were transfected into COS or 293 cells by using calcium phosphate. Two days later, the cells were trypsinized, plated in dilutions, and selected with hygromycin (200 μg/ml) for 10 to 14 days until colony formation. The colonies were directly analyzed for expression of FLAG epitope-tagged protein by immunohistochemistry in situ after fixation with 50% methanol–50% acetone for 5 min. Anti-FLAG antibody was used at dilutions of 1/200 to 1/500 with other reagents as described previously (33). Colonies were visualized following staining with aqueous hematoxylin.

Colocalization and apoptosis assay. For colocalization, COS cells were plated on slides and transfected with a plasmid expressing Bcl2 and a plasmid expressing Bbp. Two days after transfection, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, rinsed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 30 min, and blocked with a solution of 2% bovine serum albumin (BSA) in PBS (PBS-BSA) for 5 min. Cells were treated with a mixture of mouse anti-Bcl2 antibody (1/100; Dako, Carpinteria, Calif.) and affinity-adsorbed rabbit anti-Bbp diluted 1/200 in PBS-BSA for 2 h. The slides were washed for 5 min in PBS and then incubated with a biotinylated anti-mouse antibody (1/200 in PBS-BSA for 1 h). The washing step was repeated, and the slides were incubated with streptavidin-Texas red (Vector Laboratories, Burlingame, Calif.) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, Ind.), each diluted 1/200 in PBS-BSA for 1 h. Appropriate control experiments did not reveal nonspecific staining by primary or secondary reagents. Staining was analyzed with a Molecular Dynamics model 2010 confocal laser scanning microscope with ImageSpace software running on a Silicon Graphics Indigo2 workstation. Images were processed and printed by using Adobe Photoshop.

For apoptosis assays, NIH 3T3 cells were plated on slides and transfected with a Bbp- or NFAT-expressing construct. The cells were fixed and stained as described above, using the rabbit anti-Bbp antibody followed by the secondary FITC-conjugated goat anti-rabbit or the anti-FLAG tag antibody followed by secondary FITC-conjugated goat anti-mouse antibody. Cells were additionally stained with Hoechst dye to visualize nuclear morphology characteristic for apoptosis. We have correlated the Hoechst staining with the TUNEL assay and find that the two assays are equivalent in detecting apoptotic NIH 3T3 cells induced by serum starvation.

Transient transfections and cell cycle analysis. A FLAG-BBP expression plasmid (10 μg) was cotransfected with a CD20 expression plasmid (1 μg) into 293 cells, using a standard calcium phosphate precipitation technique. Similar cotransfections were done with plasmids expressing β-galactosidase. The cells were harvested 3 days after transfection and stained with a FITC-labeled anti-CD20 antibody and propidium iodide as previously described for analysis of cyclin-dependent kinases in cell cycle control (51). Flow cytometry analysis was performed on a Becton Dickinson FACStar. Cell cycle analysis was done on 10,000 CD20-positive cells for each sample.

Nucleotide sequence accession number. The BBP/53BP2 sequence has been deposited in GenBank under accession number U58334.

TABLE 1. Specificity testing of BBP interactions with multiple genes in the yeast two-hybrid system[a]

Tester strain containing:	Growth on 3-AT plates	In situ β-galactosidase assay
BCL2		
Truncated at *Bam*HI site	++	++
Full length	++	++
p53 gene	+++	+++
LYL1	−	−
P49 (polymerase α fragment) gene	−	−
Lamin gene	−	−
SNF1	−	−
HRX (C terminus)	−	−
Polymerase δ gene	−	−
EVE		
Homeodomain	−	−
Full length	−	−
UBX homeodomain	−	−
SNF1 × *SNF4*	++++	++++

[a] Growth on AT plates and β-galactosidase activity are scored as a range from − (no growth on AT and no β-galactosidase activity) to ++++ (activity generated by the strong positive control).

RESULTS

Identification of a protein that interacts with Bcl2 and p53. To identify novel proteins with the ability to interact with Bcl2, a yeast two-hybrid screen was conducted with a bait consisting of the first 188 amino acids of Bcl2 fused to the Gal4 DBD (see Materials and Methods). Two plasmids (pAD-BBP^b and pAD-BBP^c) were obtained from the initial library screening and subsequently shown to contain slightly different versions of the same human cDNA (see below). To test for the specificity of the interaction with Bcl2, one of the plasmids (pAD-BBP^c) was introduced into yeast strains expressing the Gal4 DBD fused with a variety of heterologous proteins, including p53, Lyl1, Hrx, Snf1, DNA polymerases, and homeodomain proteins. Unexpectedly, pAD-BBP^c interacted not only with DBD-Bcl2 but also with DBD-p53, although no interactions were detected with any of the other heterologous Gal4 DBD fusion proteins tested (Table 1). Interestingly, AD-Bbp interacted more strongly with DBD-p53 than with DBD-Bcl2, as evidenced by the fact that pAD-BBP/DBD-p53 colonies grew more quickly on -AT plates and turned blue sooner with in situ 5-bromo-4-chloro-3-indolyl phosphate-β-D-galactopyranoside (X-Gal) assays. The observed interaction of AD-Bbp with DBD-Bcl2 was not the result of an unusual conformation specific to the fragment of Bcl2 present in the latter, since a similar interaction was detected between AD-Bbp and a Gal4 DBD fusion protein containing full-length Bcl2 (Table 1). These studies suggested that the protein expressed by pAD-BBP specifically interacted with both Bcl2 and p53 under conditions of the yeast two-hybrid assay.

Bbp is identical to 53BP2, a putative p53-binding protein. Nucleotide sequences were determined for the cDNA inserts in both pAD-BBP plasmids. These data showed that both represented overlapping fragments of the same cDNA, with minor differences at their 5′ and 3′ ends. The cDNA insert in pAD-BBP^b begins 48 bp upstream of the insert in pAD-BBP^c and ends at a poly(A) sequence. The insert in pAD-BBP^c contains a 48-bp sequence in the open reading frame that is missing in pAD-BBP^b but present in all subsequently analyzed BBP cDNA clones (see below). Since BBP interacted with p53 in the yeast two-hybrid system, we compared the sequence of our clone with the sequence of a cDNA fragment known as 53BP2 previously isolated in a two-hybrid screen using p53 as the bait and found a direct match in the coding region. BBP/53BP2 codes for a protein with two adjacent ankyrin repeats followed by an SH3 domain (Fig. 1). Ankyrin repeats are found in many proteins and are believed to modulate protein-protein interactions. SH3 domains are also involved in protein-protein interactions by binding to proline-rich sequences and are implicated in cellular localization of proteins (4).

Various subclones of pAD-BBP^c were constructed in the pACTII vector in order to identify regions responsible for interaction with Bcl2 and p53. The expression of each construct was confirmed by Western blotting. A clone containing the ankyrin repeats and SH3 domain interacted with both p53 and Bcl2, whereas shorter clones containing either the ankyrin repeats or SH3 domain did not significantly interact, as assessed by growth in the presence of 3-AT (Fig. 1). These data suggested that both motifs were required for specific interaction with either Bcl2 or p53.

Specific interaction of Bbp/53BP2 with Bcl2 and p53 in vitro. The ability of Bbp/53BP2 to interact with Bcl2 and p53 was further studied in vitro by using GST and MBP fusion proteins (54). A fragment of Bbp containing the ankyrin repeats and SH3 domain was expressed as a GST fusion protein in *Escherichia coli*. Fragments of Bcl2, p53, and paramyosin were expressed as MBP fusion proteins in *E. coli* (see Mate-

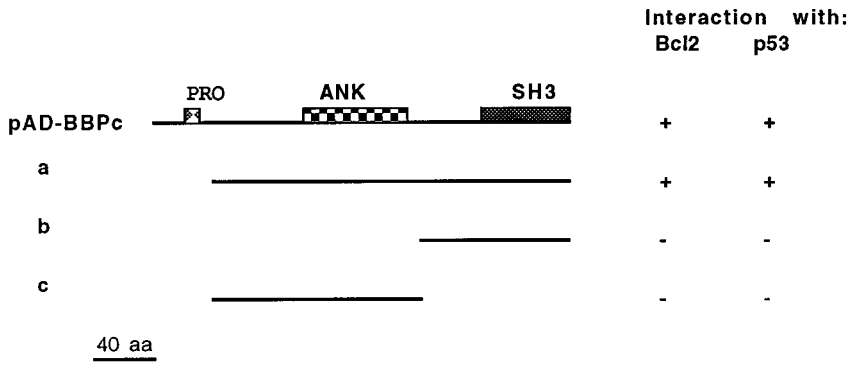

FIG. 1. Bbp interaction with Bcl2 or p53 requires ankyrin repeats and the SH3 domain. Various subclones of pAD-BBP^c (AD-Bbp fusion) were constructed in pACTII and assessed for the ability to confer 3-AT resistance to Y153/GAL4-BCL2 and Y153/GAL4-p53. Shown are structures of pAD-BBP^c and shorter subclones containing both ankyrin (ANK) repeats and the SH3 domain (a), only the SH3 domain (b), and only ankyrin repeats (c). +, growth on medium with 3-AT; −, lack of growth. Constructs were verified by DNA sequencing of the ACT domain/insert junction and confirmed by Western blotting for protein expression. PRO, proline-rich region; aa, amino acids.

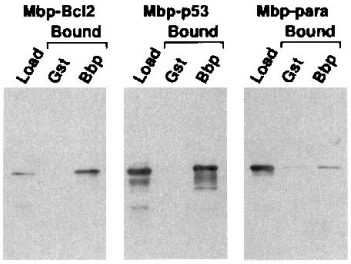

FIG. 2. GST-Bbp binds to MBP-Bcl2 and MBP-p53 in vitro. Crude extracts of GST or GST-Bbp were mixed with crude extracts of various MBP fusions or MBP and processed as described in the text. Lanes: Load, 1% of MBP fusion protein extracts incubated with GST or GST-Bbp; Gst or Bbp, MBP fusion proteins bound to GST or GST-Bbp. para, paramyosin.

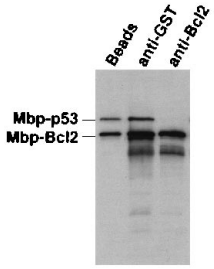

FIG. 4. Bbp does not bind Bcl2 and p53 simultaneously. GST-Bbp was incubated with a mixture of MBP-p53 and MBP-Bcl2. GST-Bbp and bound proteins were purified on glutathione resin and eluted with glutathione. Complexes were immunoprecipitated with either an anti-GST or anti-Bcl2 antibody and then subjected to Western blotting with an anti-MBP antibody. Dashes indicate positions of MBP-Bcl2 and MBP-p53.

rials and Methods). Bacterial lysates containing GST or GST-Bbp were mixed with lysates containing various MBP fusion proteins and incubated, and the GST-containing complexes selectively recovered by using glutathione-agarose beads. Protein complexes bound to the glutathione beads were subjected to polyacrylamide gel electrophoresis (PAGE) and then visualized by Coomassie staining and Western blot analysis using an anti-MBP antibody. Both MBP-Bcl2 and MBP-p53 proteins bound GST-Bbp but not GST alone (Fig. 2). In contrast, significant amounts of MBP-paramyosin did not bind to GST or GST-Bbp (Fig. 2). As a further control, MBP did not bind to either GST or GST-Bbp (data not shown). Although we had low but reproducible binding of GST-Bbp to MBP-p53, Iwabuchi et al. (24) reported that up to 84% of input full-length p53 bound to GST-53BP2. These data suggested that Bbp specifically interacted with Bcl2 and p53 in vitro as well as in the yeast two-hybrid assay.

Competitive binding of Bcl2 and p53 to Bbp. Since both Bcl2 and p53 bound Bbp in vivo in yeast cells and in vitro, we attempted to determine if they could compete for binding to Bbp. For these experiments, a limiting amount of GST-Bbp was incubated with excess MBP-Bcl2 for 1 h, increasing amounts of MBP-p53 were added for an additional hour, and then complex composition was analyzed. Alternatively, GST-Bbp and MBP-p53 complexes were formed, then increasing amounts of MBP-Bcl2 were added for 1 h, and complex composition was analyzed. When GST-Bbp/MBP-Bcl2 complexes were preformed, p53 could not effectively participate in complex formation by displacing significant amounts of bound MBP-Bcl2 (Fig. 3). However, when GST-Bbp/MBP-p53 complexes were preformed, MBP-Bcl2 could participate in complex formation by displacing some bound MBP-p53 (Fig. 3). Similar competition experiments were also performed with increasing amounts of MBP, which did not bind to GST-Bbp or displace prebound MBP-Bcl2 or MBP-p53 (data not shown).

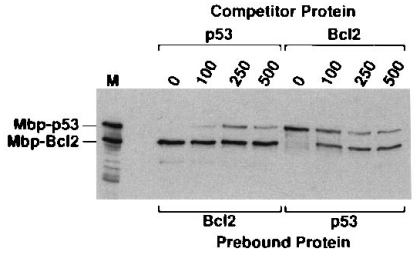

FIG. 3. Bcl2 inhibits binding of p53 to Bbp. MBP-Bcl2 prebound to GST-Bbp (Bcl2 prebound protein) was challenged with increasing amounts of MBP-p53 competitor protein. Binding of MBP-p53 is inhibited. MBP-p53 prebound to GST-Bbp (p53 prebound protein) was challenged with increasing amounts of MBP-Bcl2 competitor protein. Addition of MBP-Bcl2 displaces some bound p53. Complexes were isolated and visualized by Western blotting with an anti-MBP antibody. Lane M contains a mixture of MBP-Bcl2 and MBP-p53. Amounts of competitor proteins are indicated in approximate microgram quantities.

These data suggested that there may be only one binding site on BBP for either Bcl2 or p53, preventing their simultaneous binding, in which case trimolecular complexes involving Bbp, Bcl2, and p53 could not form. To test this possibility, GST-Bbp was incubated with a mixture of MBP-p53 and MBP-Bcl2. GST-Bbp and bound proteins were purified on glutathione resin and eluted with glutathione. Immunoprecipitation with an anti-GST antibody resulted in coprecipitation of MBP-Bcl2 and MBP-p53 (Fig. 4). Immunoprecipitation with an anti-Bcl2 antibody precipitated MBP-Bcl2 but not MBP-p53, indicating that GST-Bbp/MBP-Bcl2 complexes do not contain MBP-p53 (Fig. 4). It is possible that the anti-Bcl2 antibody would have disrupted Bcl2-Bbp-53BP2 complexes had they existed. Control experiments demonstrated that the anti-Bcl2 antibody did not disrupt Bcl2-Bbp complexes (data not shown). A parallel analysis using an anti-p53 antibody (pAb240) was not possible since it cross-reacted slightly with MBP-Bcl2 (data not shown).

Isolation of full-length clones of BBP. The BBP cDNA fragment obtained from yeast two-hybrid screening was used as a hybridization probe to isolate full-length BBP cDNAs. The largest cDNAs of approximately 4.5 kb matched the size of BBP mRNA detected by Northern (RNA) blot analysis (Fig. 5A). Nucleotide sequence analyses showed the presence of an *Alu* repeat element in the 5′ untranslated region followed by two potential initiating ATG codons at nucleotide positions 571 and 757. The latter was demonstrated to be the preferred initiation codon on the basis of in vitro translation of truncated BBP cDNAs (Fig. 5B). These data predict a BBP-encoded protein of 1,005 amino acids and 113 kDa, although its migration in SDS-PAGE is noted to be anomalously large at 150 kDa (Fig. 5B).

The Bbp sequence (Fig. 6) predicted from the full-length cDNA sequence was used as a query for database homology searches using the BLAST program (2). These searches showed that Bbp carboxy-terminal portions were similar to those of previously reported proteins containing either SH3 or ankyrin repeat motifs. Highest similarity scores were obtained for the SH3 motif in GRB2 and the ankyrin motif in human ankyrin. In addition, the first 200 amino acids of Bbp showed limited similarity to the alpha-helical rod domain of various myosin heavy chains. Further inspection revealed that this region of Bbp is composed of heptad repeat units which can theoretically assume an alpha-helical conformation, as determined by the

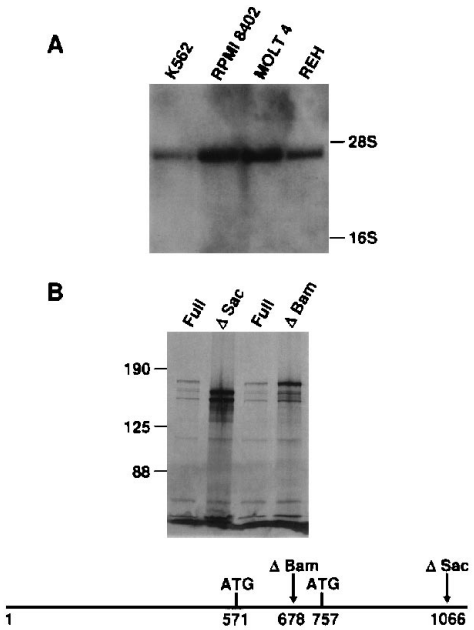

FIG. 5. (A) Northern blot of BBP message. Poly(A)$^+$ RNA was obtained from cell lines K562 (erythroid), RPMI 8402 (early T cell), MOLT 4 (mature T cell), and REH (precursor B cell). (B) In vitro transcription-translation of BBP cDNA to define the initiator ATG. The schematic diagram of the 5′ end of BBP shows potential ATG starts and restriction enzyme sites. Lanes: Full, full length; ΔSacI, deleted at the *Sac*I site; ΔBam, deleted at the *Bam*HI site. Molecular weight markers in kilodaltons are indicated on the left.

Chou-Fasman prediction algorithm (9). The first 100 amino acids of this region are rich in glutamine and have limited homology to glutamine-rich proteins, including transcription factors. Additionally, visual inspection revealed a proline-rich region from amino acids 743 to 752 (YPPYPPPPYP) which fits the consensus for an SH3 domain binding site (38).

Cytoplasmic localization of Bbp/53BP2. An expression vector was used to place a FLAG epitope tag on the 5′ end of Bbp. The construct was transfected into COS cells. Western blotting with an anti-FLAG antibody detected proteins of approximately 150 and 140 kDa (Fig. 7). Two different rabbit anti-Bbp antibodies detected the same protein bands with signal intensities similar to those of the anti-FLAG antibody, suggesting that the anti-Bbp polyclonal antibody compares favorably with the highly sensitive and specific monoclonal anti-FLAG antibody. These results are consistent with the molecular weight as determined by in vitro translation. The lower band may represent a degradation product or a protein resulting from an internal translation initiation. A construct which expresses native nontagged Bbp also produced a protein of 150 kDa, as determined by Western blotting with the rabbit anti-Bbp antibodies (data not shown).

The FLAG-tagged construct was also transfected into COS cells and detected with an antibody directed against the FLAG epitope by using immunohistochemistry and immunofluorescence. The protein appeared cytoplasmic, being mostly absent in the nucleus. By immunofluorescence analysis, a punctate vesicular pattern was noted (Fig. 8a). An anti-Bbp rabbit polyclonal antibody gave a virtually superimposable pattern (data not shown). A similar cytoplasmic pattern was seen in transiently transfected Rat1, CV-1, 293, and NIH 3T3 cells (data not shown).

To determine if Bcl2 and Bbp/53BP2 colocalized, COS cells were cotransfected with DNA constructs expressing each protein and analyzed by confocal microscopy. Bcl2 protein (red fluorescence in Fig. 8b and e) is detected mainly in the perinuclear region, endoplasmic reticulum, and mitochondrial membranes, whereas Bbp/53BP2 is more widely distributed throughout cytoplasmic structures (green fluorescence in Fig. 8a and d). Areas of colocalization between the two antigens are shown in the overlay as a yellow color (Fig. 8c and f) and appear mostly in the perinuclear region.

```
MDLTLAELQE MASRQQQQIE AQQQLLATKE QRLKFLKQQD    40
QRQQQQVAEQ EKLKRLKEIA ENQEAKLKKV RALKGHVEQK    80
RLSNGKLVEE IEQMNNLFQQ KQRELVLAVS KVEELTRQLE   120
MLKNGRIDSH HDNQSAVAEL DRLYKELQLR NKLNQEQNAK   160
LQQQRECLNK RNSEVAVMDK RVNELRDRLW KKKAALQQKE   200
NLPVSSDGNL PQQAASAPSR VAAVGPYIQS STMPRMPSRP   240
ELLVKPALPD GSLVIQASEG PMKIQTLPNM RSGAASQTKG   280
SKIHPVGPDW SPSNADLFPS QGSASVPQST GNALDQVDDG   320
EVPLREKEKK VRPFSMFDAV DQSNAPPSFG TLRKNQSSED   360
ILRDAQVANK NVAKVPPPVP TKPKQINLPY FGQTNQPPSD   400
IKPDGSSQQL STVVPSMGTK PKPAGQQPRV LLSPSIPSVG   440
QDQTLSPGSK QESPPAAAVR PFTPQPSKDT LLPPFRKPQT   480
VAASSIYSMY TQQQAPGKNF QQAVQSALTK THTRGPHFSS   520
VYGKPVIAAA QNQQQHPENI YSNSQGKPGS PEPETEPVSS   560
VQENHENERI PRPLSPTKLL PFLSNPYRNQ SDADLEALRK   600
KLSNAPRPLK KRSSITEPEG PNGPNIQKLL YQRTTIAAME   640
TISVPSYPSK SASVTASSES PVEIQNPYLH VEPEKEVVSL   680
VPESLSPEDV GNASTENSDM PAPSPGLDYE PEGVPDNSPN   720
LQNNPEEPNP EAPHVLDVYL EEYPPYPPPP YPSGEPEGPG   760
EDSVSMRPPE ITGQVSLPPG KRTNLRKTGS ERIAHGMRVK   800
FNPLALLLDS SLEGEFDLVQ RIIYEVDDPS LPNDEGITAL   840
HNAVCAGHTE IVKFLVQFGV NVNAADSDGW TPLHCAASCN   880
NVQVCKFLVE SGAAVFAMTY SDMQTAADKC EEMEEGYTQC   920
SQFLYGVQEK MGIMNKGVIY ALWDYEPQND DELPMKEGDC   960
MTIIHREDED EIEWWWARLN DKEGYVPRNL LGLYPRIKPR  1000
QRSLA                                        1005
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FIG. 6. Predicted amino acid sequence of BBP-encoded protein. A potential alpha-helical region is underlined, a proline-rich region is indicated by a wavy underline, ankyrin repeats are double underlined, and the SH3 domain is boxed. The start sites of the clones isolated in the yeast two-hybrid screen are shown: pAD-BBP^b, closed arrowhead; pAD-BBP^c, open arrowhead.

Stable cell lines expressing Bbp cannot be isolated. Trans-

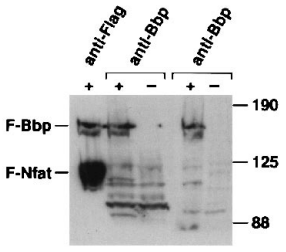

FIG. 7. Western blot analysis of Bbp produced in COS cells. Cells were cotransfected with constructs expressing FLAG-tagged NFAT and BBP (+) or mock transfected (−), and protein extracts were analyzed by Western blotting with an anti-FLAG tag antibody or two different rabbit anti-Bbp antibodies. Sizes are indicated in kilodaltons.

FIG. 8. Partial colocalization of Bbp/53BP2 with Bcl2 demonstrated by confocal microscopy. (a and d) Bbp shown in green; (b and e) Bcl2 shown in red; (c and f) overlay showing colocalization in yellow.

fection of various cell lines (COS, 293, and NIH 3T3) with an Epstein-Barr virus nuclear antigen and ori plasmid (CEP4) carrying FLAG epitope-tagged BBP gave rise to colonies after approximately 7 to 14 days of selection; however, none of the cells in the colonies expressed detectable levels of Bbp, as determined by in situ immunohistochemical staining and Western blotting with the anti-FLAG tag antibody (data not shown). To quantitate BBP transfection efficiency and inability to form stably expressing cell lines, CEP4, CEP-FBBP (CEP4 containing FLAG-tagged BBP), CEP-FNFAT (CEP4 containing FLAG-tagged NFAT as a control protein), and CEP-revFBBP (CEP4 containing a FLAG-tagged BBP DNA fragment cloned in reverse orientation) were transfected into COS cells and analyzed for colony formation and epitope expression. Approximately equivalent numbers of cells were transfected in the case of CEP-FBBP and CEP-FNFAT, as assessed by immunohistochemistry of individual cells at day 2 after transfection (Table 2). After growth in selection medium, CEP-FBBP had the lowest number of colonies per microgram of transfected DNA and resulted in no positive colonies. CEP-FNFAT gave rise to positively staining colonies (Table 2). The construct containing BBP DNA in the reverse orientation, which should not express Bbp, gave many colonies, suggesting that the BBP DNA fragment by itself was not toxic to the transfected cells. These results indicated that expression of FLAG-tagged BBP was toxic to cells, whereas expression of FLAG-tagged NFAT was not. We obtained similar results with human embryonic kidney 293 and NIH 3T3 cells (data not shown), which were used in further experiments.

Bbp does not induce apoptosis in transiently transfected NIH 3T3 cells. To determine if expression of BBP induced apoptosis, we transfected NIH 3T3 cells. These cells have been used extensively by other researchers studying apoptosis-inducing proteins. The transfected cells were stained for Bbp/53BP2 antigen and nuclear morphology as described in Materials and Methods. Three days after transfection, many Bbp-staining cells were rounded and appeared unhealthy (Fig. 9A and C) but did not show any evidence of nuclear condensation

TABLE 2. Analysis of transient and stable transfectants[a]

Construct	% Cells positive for FLAG tag at day 2 (200 cells counted)	No. of colonies/μg of transfected DNA at day 14	% Colonies positive for FLAG tag at day 14
CEP-FBBP	6	34	0
CEP-revFBBP[b]	NT[c]	>500	0
CEP4 alone	NT	>500	0
CEP-FNFAT	8.5	107	45

[a] Similar results were obtained from multiple experiments.
[b] Contains the BBP DNA fragment in reverse orientation and does not express Bbp.
[c] NT, not tested because the construct does not produce epitope-tagged protein.

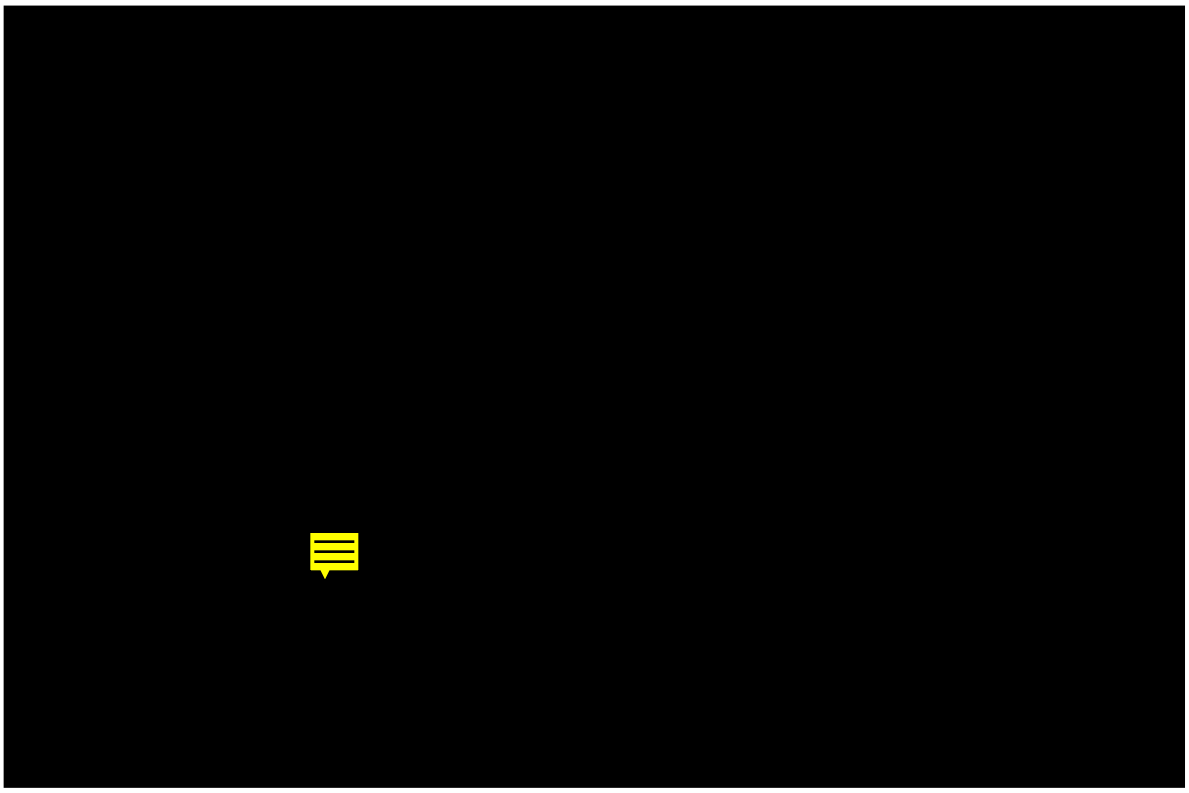

FIG. 9. Bbp transfection does not cause apoptosis in NIH 3T3 cells. (A and C) Bbp-transfected cells; (B and D) nuclear morphology of Bbp-transfected cell.

or fragmentation characteristic of apoptotic cells (Fig. 9B and D). We have correlated Hoechst staining with the tunnel assay and find that they detect apoptotic NIH 3T3 cells equally well. In parallel experiments, cells transfected with NFAT appeared to have normal cytoplasmic and nuclear morphology (data not shown).

Bbp impedes cell cycle progression at G_2/M. To determine if expression of BBP perturbed the cell cycle, we cotransfected 293 cells with plasmids expressing CD20–β-galactosidase and CD20–FLAG-tagged Bbp. Cells were analyzed for CD20 and cell cycle distribution 3 days after transfection. Cell cycle distribution was determined for cells expressing high amounts of CD20 and presumably coexpressing β-galactosidase or Bbp.

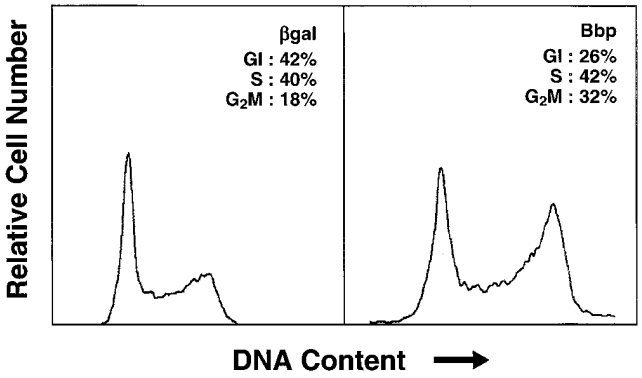

FIG. 10. BBP induces an accumulation at G_2/M of the cell cycle. Human 293 cells were cotransfected with CD20 and plasmids expressing β-galactosidase (βgal) or FLAG-tagged Bbp and analyzed as described in the text.

Significantly more cells were detected at G_2/M in FLAG-tagged Bbp transfectants (Fig. 10), suggesting that Bbp induces an accumulation of cells at G_2/M.

DISCUSSION

We have used the yeast two-hybrid system to isolate cDNAs coding for a protein that interacts with Bcl2. This protein (Bbp) has not been isolated in previous attempts to isolate Bcl2-interacting proteins by using either yeast two-hybrid or biochemical techniques. In our two-hybrid screen, we did not isolate any of the other genes which have been shown to interact with Bcl2 by coimmunoprecipitation or by the yeast two-hybrid system. These include genes encoding Bcl2 itself, R-Ras p23, Bax, Bcl-x_L, Bcl-x_S, Mcl-1, Nip1, Nip2, Nip3, Bak, and Bad (5, 6, 8, 16, 17, 26, 35, 39, 56–58). The initial fragment of Bcl2 that we used as the bait for our two-hybrid screening lacked 51 amino acids from the carboxy-terminal end, some of which may be required for these interactions. Additionally, appropriate cDNAs for these Bcl2-interacting proteins may not be well represented in the oligo(dT)-primed cDNA library that we used. The latter possibility is quite likely since screening of this library with Bcl-x_L as bait also did not yield any of the foregoing proteins known to interact with Bcl2 but screening of a different library did (unpublished observations).

Our studies demonstrate that the interaction of Bbp with Bcl2 requires the ankyrin repeats and SH3 domain. Both of these protein motifs have previously been shown to mediate protein-protein interactions. Unexpectedly, we found that Bbp also interacts with p53 in the yeast two-hybrid system. The region of Bbp responsible for this interaction includes both the ankyrin repeats and SH3 domain as is the case for Bcl2. Our in

vitro binding studies reveal that a GST-Bbp fusion protein produced in *E. coli* can interact with both MBP-Bcl2 and MBP-p53 but not with control proteins. Experiments designed to test if both Bcl2 and p53 can bind simultaneously to Bbp indicate that Bbp can bind either Bcl2 or p53 but not both at the same time. Competition experiments indicate that Bcl2 can prevent the binding of p53 to Bbp and can displace p53 after it is bound to Bbp.

Using p53 as bait in a two-hybrid screen, Iwabuchi et al. isolated a gene fragment, 53BP2, which corresponds to BBP (24). Their evidence indicates that the 53BP2/p53 complex cannot bind DNA, and therefore 53BP2-bound p53 could potentially be transcriptionally inactive. They and Thukral et al. found that 53BP2 binds to wild-type but not some mutant forms of p53, suggesting that binding is dependent on p53 conformation (49). Their studies were performed with a fragment of the gene initially isolated through the yeast two-hybrid screen.

To elucidate the functions of this protein, we cloned the entire BBP cDNA and showed that the predicted protein contains an alpha-helical repeat at its amino terminus. Such regions may mediate protein-protein interactions. Thus, Bbp/53BP2 contains at least three motifs which could potentially mediate interactions with other proteins. Interestingly, Bbp/53BP2 has two areas of homology to myosin, which also contains an SH3 domain and an alpha-helical region repeat. We have determined that Bbp/53BP2 is localized in the cytoplasm of cells in a punctate granular/vesicular pattern. This distribution partially overlaps that of Bcl2, which is primarily localized to membranes of the endoplasmic reticulum, mitochondria, and nuclear envelope. On the other hand, p53 is localized primarily in the nucleus. Bbp is made in small amounts in transiently transfected cells and appears to be in an insoluble fraction that can be extracted only with buffers containing ionic detergents such as SDS (unpublished observations). We have tried without success to coimmunoprecipitate Bcl2 or p53 with Bbp in mammalian cells. Our inability to coimmunoprecipitate the proteins in SDS-containing buffers suggests that the interactions (if present) are probably not strong. However, many biologically relevant interactions, such as the interaction between Bcl2 and Bax, are disrupted in SDS-containing detergents (33).

Although we have been able to express Bbp/53BP2 transiently in many different types of cell lines, we have been unable to obtain stable long-term expression of Bbp/53BP2. Our data suggest that Bbp/53BP2 overexpression is toxic to cells. To investigate this possibility further, we analyzed the ability of BBP to induce apoptosis in transfected NIH 3T3 cells. Although the cells appeared rounded, there was no evidence for apoptosis as judged from nuclear condensation or fragmentation. Since apoptosis did not seem to account for Bbp toxicity, we also performed cell cycle analyses on 293 cells transiently transfected with BBP. We found that overexpression of BBP caused the accumulation of some transfected cells at the G_2/M phase of the cell cycle.

Recently, p53 has been shown to induce a G_2/M arrest as well as a G_1 arrest through an unknown mechanism (1, 44). Additionally, p53 has been implicated as a component of a spindle checkpoint that ensures maintenance of diploidy (12). Bbp may induce a G_2/M accumulation through an enhancement of the G_2/M arrest activity of p53 by direct physical interaction with p53. Although high-level overexpression of Bbp can perturb the cell cycle, these results do not imply that the normal role of Bbp is to modulate the cell cycle.

Although the G_2/M accumulation is not a profound effect, it may explain our inability to produce stable cell lines expressing Bbp/53BP2. Interestingly, BBP mRNA is readily detectable, but we have not detected Bbp/53BP2 protein in any of a wide variety of human cell lines and tissues (unpublished data), suggesting that its expression is exceedingly low or limited to conditions that were not examined in our studies. Our inability to produce stable cell lines or to detect significant amounts of Bbp in normal cells are concordant and suggest that Bbp/53BP2 may be a potent modulator of cell growth or survival whose expression is controlled at the posttranscriptional level.

Although we and others have isolated Bbp/53BP2 by using two different baits in the yeast two-hybrid system, the biological significance of these interactions has not yet been clarified. Wild-type p53 is sequestered in the cytoplasm of the majority of undifferentiated neuroblastomas and some breast cancers (30, 31). Since Bbp/53BP2 is a cytoplasmic protein, it may be involved in the sequestration process. Future experiments will be directed toward elucidating the mechanism of Bbp-induced G_2/M arrest with the aim of determining the relevance of Bbp/53BP2 for Bcl2 and p53 function.

ACKNOWLEDGMENTS

This work was supported by grant CA42971 from the National Institutes of Health. L.N. was a postdoctoral fellow of the Howard Hughes Medical Institute and is a recipient of an Amgen/ASH Scholar Award. M.L.C. is a scholar of the Leukemia Society of America.

We thank Stephen Elledge for making plasmids available before publication, Stan Fields and Bin Li for exchange of materials, Brad Johnson, Teresa Wang, and Min Cui for plasmid constructs, and Stephen Smith for cell lines. The comments from various members of the Cleary laboratory, including Kevin Smith, Stephen Hunger, Jeffrey Rubnitz, and David LeBrun, were greatly appreciated.

ADDENDUM IN PROOF

53BP2 also interacts with protein phosphatase 1 (N. R. Helps et al., FEBS Lett. **377:**295–300, 1995).

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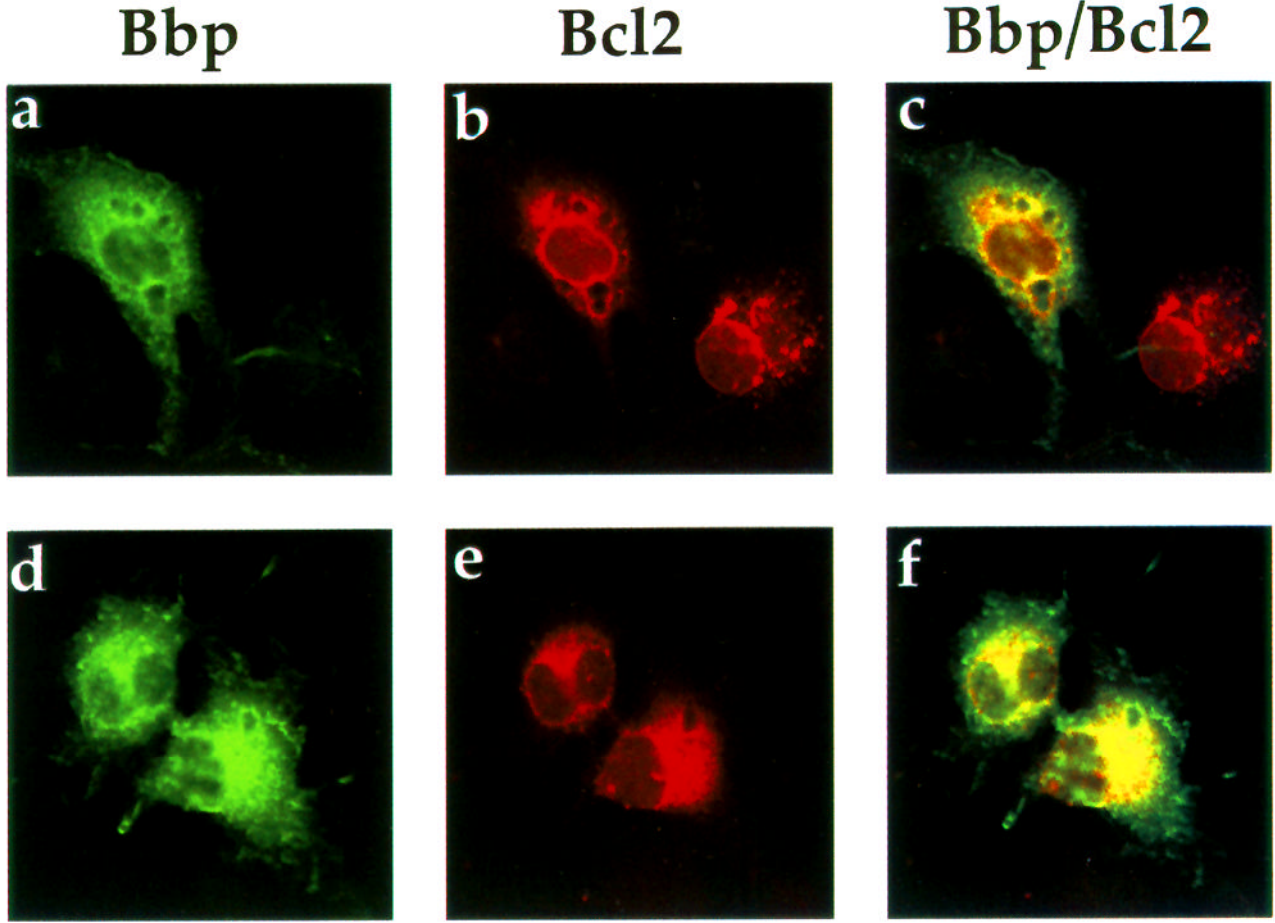
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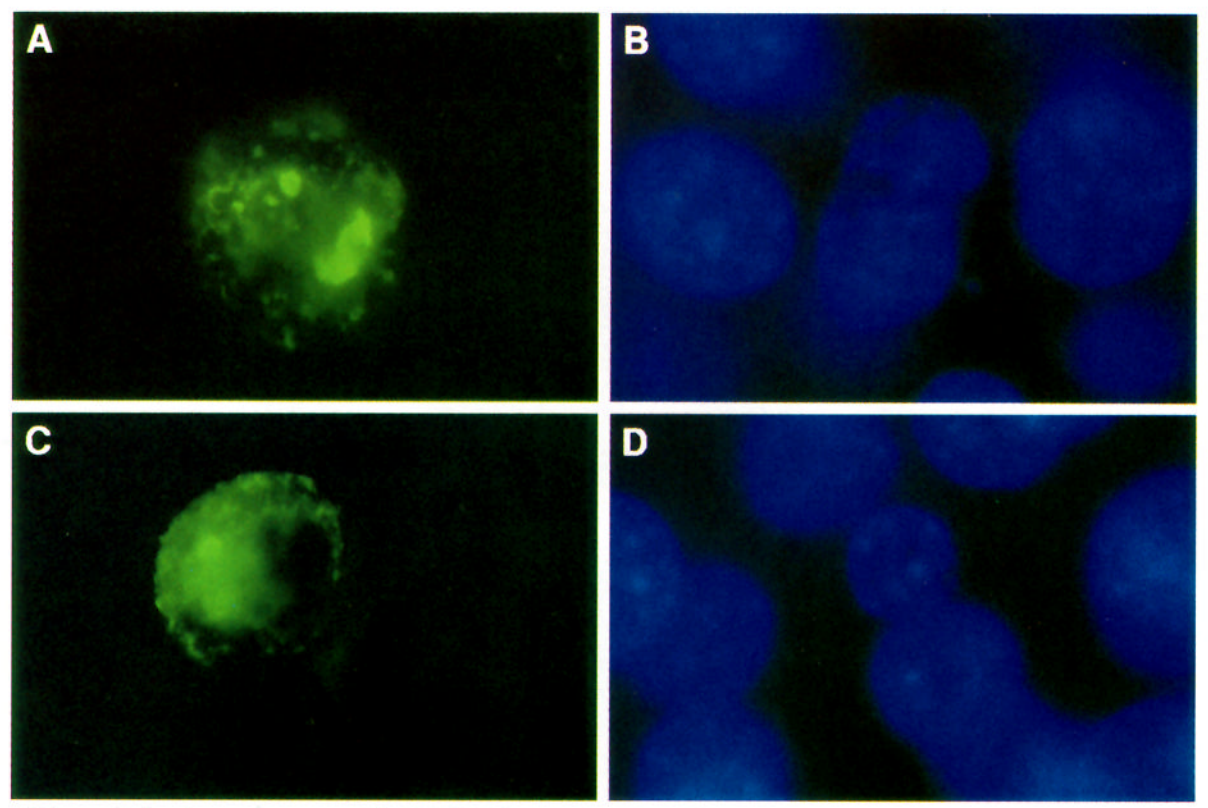
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