Identification and Characterization of BCL-3-binding Protein

IMPLICATIONS FOR TRANSCRIPTION AND DNA REPAIR OR RECOMBINATION*

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A putative oncogene bcl-3 was originally identified and cloned at the breakpoint in the recurring chromosome translocation t(14;19) found in some cases of B cell chronic lymphocytic leukemia. Studies of bcl-3-deficient mice demonstrated a critical role for bcl-3 in the development of a normal immune response and the formation of germinal centers in secondary lymphoid organs. However, the molecular mechanism that underlies B cell leukemogenesis and the knockout mouse phenotype remains unclear. Here we have identified and characterized BCL-3-binding protein (B3BP) as a protein interacting specifically with the bcl-3 gene product (BCL-3) by a yeast two-hybrid screen. We found that B3BP associates with not only BCL-3 but also p300/CPBP histone acetyltransferases. The N-terminal region of B3BP that contains the ATP-binding site is important for the interaction with BCL-3 and p300/CPBP. Homology searches indicate that the ATP-binding region of B3BP, which resembles a typical Walker-type ATP-binding P-loop, most resembles that of 2',3'-cyclic nucleotide 3'-phosphodiesterase of mammals and polynucleotide kinase of T4 bacteriophage. In fact B3BP shows intrinsic ATP phosphodiesterase of mammals and polynucleotide kinase of T4 bacteriophage. In fact B3BP shows intrinsic ATP binding and hydrolyzing activity. Furthermore, we demonstrated that B3BP is a 5'-polyadenylate kinase. We also found a small MutS-related domain, which is thought to be involved in the DNA repair or recombination reaction, in the C-terminal region of B3BP, and it shows nicking endonuclease activity. These observations might help to gain new insights into the function of BCL-3 and p300/CPBP, especially the coupling of transcription with repair or recombination.

bcl-3 was originally identified at the breakpoint in the t(14;19) chromosome translocation in some cases of chronic B cell lymphocytic leukemia and shown to be up-regulated transcriptionally in peripheral blood lymphocytes from patients with the corresponding translocation. Therefore, its involvement in the pathogenesis of chronic B cell lymphocytic leukemia has been strongly suggested (1). It was also reported that bcl-3 gene expression is induced by many cell growth- or survival-promoting factors in lymphocytic cell lines (2–4), suggesting the close involvement of bcl-3 in cell proliferation and survival. Subsequent studies showing a correlation of bcl-3 induction with mouse skin carcinogenesis (5), human breast cancer (6), and hepatocyte proliferation (7) have indicated its involvement in carcinogenesis and the growth of cells other than B lymphocytes. In fact, ectopic expression of bcl-3 blocked interleukin-4 deprivation-induced apoptosis of a T cell line in vitro and also increased the survival rate of activated T cells in vivo (8, 9). Transgenic mice in which bcl-3 is expressed under the control of an Ig heavy chain enhancer showed an expansion of the B cell population (10). Nevertheless, there is no experimental evidence of a role for bcl-3 in cell transformation.

Extensive biochemical study has revealed some of the molecular functions of bcl-3. First, amino acid sequence alignment showed that BCL-3 contains seven repeats of an ankyrin-like unit and belongs to the IxB family of proteins, which modulate the DNA binding activity and subcellular localization of the transcription factor NF-κB (11). Subsequently, it was demonstrated that BCL-3 physically associates with the p50 and p50B homodimers of NFKB1 and NFKB2, respectively, and confers transcriptional activation to the otherwise inert complex; hence it functions as a transcriptional co-activator (12, 13). We have previously demonstrated that BCL-3 induces the nuclear translocation of the p50 homodimer generated via re-organization from cytoplasmic p50/p105 (14). This BCL-3-induced p50 homodimer formation has been observed in vivo; that is, ectopic expression of BCL-3 in thymocytes induced the formation of the p50 homodimer (15), and stimulation of cultured T cells with interleukin-9 leads to the induction of BCL-3 expression, which is followed by p50 homodimer generation (3). Moreover, p50 homodimer has been implicated in cell survival or the inhibition of apoptosis (3, 16). Subsequently, it has been shown that BCL-3 interacts with general transcription factors (17), other transcriptional co-activators (18, 19), and also other DNA-binding factors (17, 18), all of which indicates a general role for BCL-3 in the transcriptional activation. Recently, it has also been demonstrated that a putative BCL-3 ortholog of Caenorhabditis elegans interacts with MRT-2 cell cycle checkpoint protein and indeed is involved in the DNA damage response by protein-protein interaction screening combined with a large scale phenotypic analysis (20).

Above all, knockout mouse studies gave rise to important information on the biological role of BCL-3 (21–23). The BCL-3-deficient mouse was susceptible to certain kinds of pathogens. Antigen-specific antibody production was severely impaired because germinal center formation in secondary lymphoid organs was markedly inhibited. Such a phenotype was at least to some extent similar to that of NFKB1 and NFKB2 knockout mice (23–26), suggesting physiological significance of the interaction of BCL-3 with these proteins. During the development of germinal centers, B cell-specific genetic recombination of the Ig gene, class switch recombination, and somatic hypermutation proceed to produce a large amount of Ig

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that has a much higher affinity for the antigen. One hypothesis is that BCL-3 directly regulates class switch recombination and somatic hypermutation because these genetic alterations have a close correlation with the transcriptional activation of the Ig gene itself and the intrinsic switch region (27–29).

In this study we identified B3BP as a protein that specifically interacts with BCL-3. It was shown that B3BP also interacts with histone acetyltransferases p300/CREB and that the ATP-binding site of B3BP is important for the association with BCL-3 or p300/CBP. Biochemical analysis revealed that B3BP has polynucleotide kinase activity to transfer a phosphate group to the 5' end of DNA and RNA substrates. Moreover, a small MutS-related (Smr) domain found in the C-terminal region of B3BP exhibits nicking endonuclease activity, which is postulated to have a role in mismatch repair or genetic recombination (30, 31). These findings suggest that B3BP plays a role connecting transcriptional activation and genetic recombination of the Ig gene.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—A fragment of mouse BCL-3 cDNA encoding amino acids 16–440 cloned into the LexA DNA-binding domain vector pBTH116 and a human spleen cDNA library cloned into the GAL4 activation domain vector pAD10 (Clontech) were sequentially used to transform L40 yeast cells according to the protocol described on the Yeast Transformation Home Page (www.umanitoba.ca/faculties/medicine/units/biochem/gists/Trafo.html). Transformants were plated on selection medium (lacking tryptophan, leucine, and histidine) containing 10 μg/ml 3-amino-1,2,4-triazole. After incubation for 7 days at 25 °C, the clones that allowed growth were identified and confirmed to express β-galactosidase. The plasmids were recovered according to Matchmaker protocols (Clontech) and retransformed into yeast cells containing either pBTH116-BCL-3 or pBTH116 empty vector. cDNA inserts from plasmids that allowed the yeast cells containing pBTH116-BCL-3 to grow on selection medium were identified and further characterized by DNA sequencing.

cDNA Cloning and Plasmid Construction—To obtain a full-length B3BP cDNA, a human T cell cDNA library carried in AzAP Express (Stratagene) was screened under stringent conditions using the cDNA of a ~4.7-kb insert isolated in the two-hybrid screen as a probe. The cDNA inserts from positive phage clones were excised in vivo to generate subclones in the pBR-CMV phagemid and confirmed by sequencing.

The ORF containing full-length B3BP cDNA was assembled on a mammalian expression vector, pEF-BOS (32). To express GST fusion proteins in Escherichia coli JM109, DNA fragments encoding amino acids 2–631, 1171–1770, 394–630, and 1688–1700 of B3BP were cloned into pGEX-4T (Amersham Biosciences) to construct pGEX-B3BP(N), pGEX-B3BP(C), pGEX-B3BP(M), and pGEX-B3BP(Smr), respectively. A cDNA encoding full-length mouse B3BP was cloned into pGEX-4T to express GST-B3BP fusion protein. cDNA fragments encoding B3BP, B3BP derivatives, and NFKB1 were subcloned into pCS2 (+) and its derivatives were expressed in 293T cells and purified using anti-HA (12CA5) and anti-FLAG (M2) monoclonal antibodies. Subsequently they were visualized with appropriate secondary antibodies conjugated with horseradish peroxidase and an ECL plus Western blotting detection system (Amersham Biosciences).

Assay for ATP Binding and Hydrolyzing Activity—B3BP-FLAG and its derivatives were expressed in 293T cells and purified using anti-FLAG M2-agarose resin as described above. For the ATP binding assay, proteins attached to the beads were suspended in buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 100 μg of leupeptin/ml, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM diithiothreitol, 1 mM sodium orthovanadate, 10% (v/v) glycerol, and 10 mM benzamidine). Subsequently, the suspensions were allowed to stand on ice for 30 min and centrifuged (356,000 × g, 10 min), and the resulting supernatant was subjected to immunoblot analysis to detect GST fusion protein. The FLAG-based NFKB1 was described previously (35).

Recombinant Proteins and in Vitro Binding Assay—All of the pGEX-based bacterial expression vectors were transformed into E. coli strain JM109, and GST fusion proteins were expressed and purified using glutathione-Sepharose resin according to the manufacturer's directions (Amersham Biosciences). For the in vitro biochemical analysis, GST, GST-B3BP(M), and GST-B3BP(Smr) were eluted from the column with buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM diethiothreitol, and 20 mM glutathione) and dialyzed against phosphate-buffered saline. Subsequently, GST-B3BP(Smr) was treated with thrombin protease, and the Smr domain was further purified by ion-exchange chromatography on a Mono S column (Amersham Biosciences). For the GST-based interaction assay, GST fusion proteins attached to glutathione matrix beads were incubated with rabbit reticulocyte lysate containing 35S-radiolabeled protein in Nonidet P-40 binding buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% (v/v) Nonidet P-40, and 10% (v/v) glycerol) for 2 h at 4 °C. The beads were subsequently washed five times with Nonidet P-40 binding buffer, and the bound proteins were fragmented by SDS-PAGE and visualized by autoradiography or Coomassie Brilliant Blue (CBB) staining. Reticulocyte lysate used in Fig. 5B was ATP-depleted by passing through the Sephadex G-50 (Amersham Biosciences) column.

Identification and Characterization of B3BP

Culture, DNA Transfection, Immunoprecipitation, and Western Blotting—293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected by the calcium phosphate method. 48 h after transfection, 293T cells (~2 × 106 cells/6-cm dish) were harvested, washed with phosphate-buffered saline, and suspended in 400 μl of the extraction buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 100 μg of leupeptin/ml, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM diithiothreitol, 1 mM sodium orthovanadate, 10% (v/v) glycerol, and 10 mM benzamidine). Subsequently, the suspensions were allowed to stand on ice for 30 min and centrifuged (356,000 × g, 10 min), and the resulting supernatant was subjected to immunoblot analysis to detect GST fusion protein. For the FLAG-based NFKB1 was described previously (35).

Assay for ATP Binding and Hydrolyzing Activity—B3BP-FLAG and its derivatives were expressed in 293T cells and purified using anti-FLAG M2-agarose resin as described above. For the ATP binding assay, proteins attached to the beads were suspended in buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, and 0.1% (v/v) Tween 20) and subjected to UV irradiation in the presence of 32P-labeled 8-azido-adenosine-5'-triphosphate (8-azido-ATP). After the washing out of the uncross-linked 8-azido-ATP, the proteins were separated by SDS-PAGE and visualized by autoradiography or CBB staining. For the ATP hydrolyzing assay, the proteins were cross-linked with 32P-labeled 8-azido-ATP and incubated in the presence or absence of 10 μg of yeast transfer RNA/ml for 60 min at 30 °C in the buffer described above. After a wash, the proteins were separated by SDS-PAGE, visualized by autoradiography or CBB staining. The amount of protein subjected to the assay was confirmed by CBB staining.

Assay for Polynucleotide Kinase Activity—Recombinant proteins, GST and GST-B3BP(M), or FLAG-tagged B3BP and its derivatives expressed in 293T cells and absorbed onto anti-FLAG resin were incubated with 20 μl (293T cell equivalent) (3 μg) of yeast transfer RNA/ml for 37 min at 37 °C in the presence of the γ32P-labeled ATP with the following substrates: single-stranded DNA, 3’OH-GGAATTCAGAATTTTAGG-CCTCCATGGGCTGCTAGTTGG-5’OH; double-stranded DNA, 40 bp annealed with 3’OH-GGAATTCAGAATTAGTTTACCCCTCGGAGCCTCCGAGCCGTTTGTACCTTCATGAGCGCCGCAAGGTCATACACATTCTG-5’OH, yeast transfer RNA (Sigma-Chemical Sciences). The products were separated on a denaturing 10% polyacrylamide gel and visualized by autoradiography. The proteins subjected to the assay were run on a NuPAGE 4–12% Bis-Tris gel with SDS-containing MES buffer (Invitrogen) and visualized by CBB staining.
Assay for Nicking Endonuclease Activity—Recombinant proteins were incubated in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 50 µg of bovine serum albumin/ml) at 37 °C for 2 h with a supercoiled circular DNA (pEF-BOS) as a substrate. The samples were separated on a 1% agarose gel and visualized by ethidium bromide staining. The proteins subjected to the assay were run on a NuPAGE 4–12% Bis-Tris gel with SDS-containing MES buffer (Invitrogen) and visualized by CBB staining.

RESULTS

Identification of B3BP as a Protein Interacting with BCL-3—We sought to identify proteins other than NFKB1 and NFKB2 that interact specifically with BCL-3. We set up a yeast two-hybrid system with full-length BCL-3 as bait and screened a GAL4 activation domain-tagged cDNA library derived from the
human spleen. Although full-length BCL-3 activates transcription substantially in yeast when tethered to DNA via the LexA DNA-binding domain, a competitive inhibitor of His3p, 3-amino-1,2,4-triazole, completely abrogated its intrinsic activity, and screening worked properly under these conditions. From a screen of $\times 10^6$ colonies, 38 clones grew on the selective medium and showed $\beta$-galactosidase activity. One clone had a 4.7-kb insert, and a protein of corresponding molecular mass ($\sim 200$ kDa) was detected by anti-GAL4 activation domain antibody in the yeast lysate (data not shown). Subsequent sequence analysis revealed that it contains a large in-frame ORF, and a database search indicated that a portion of its cDNA has been registered as KIAA1413 with unknown function. We obtained full-length cDNA by screening a human T cell cDNA library using the 4.7-kb insert as a probe. The largest clone was isolated and sequenced. It contained a 6626-bp cDNA insert and extended 132 bp 5’ of a Kozak consensus sequence for the predicted start of translation. The predicted polypeptide specified by the ORF comprised 1770 amino acids, which was calculated to be $\sim 200$ kDa. Recently, a 374-amino acid fragment near the C-terminal region of this predicted polypeptide was identified by yeast two-hybrid screening and reported as N4BP2 (Nedd4 ubiquitin ligase-binding partner 2) (36), and its full-length protein was referred to as flN4BP2. It was demonstrated that the corresponding region

**Fig. 2. In vitro binding of B3BP to BCL-3.** A, B3BP binds to BCL-3 in vitro. Equal volumes of reticulocyte lysate that contain $^{35}$S-labeled products were run on 5–20% SDS-polyacrylamide gel and visualized by autoradiography as shown in lanes 1–3 (input). Lane 1, B3BP; lane 2, BCL-3; lane 3, luciferase. Luciferase was used as a negative control in this experiment. A GST pull-down assay was performed using equal amounts of the reticulocyte lysate and purified GST (lane 4), GST-B3BP(N) (lane 5), GST-B3BP(C) (lane 6), or GST-BCL-3 (lane 7). The bound proteins were subjected to SDS-PAGE and visualized by autoradiography. Lanes 4–7 were exposed $\sim 10$ times longer than lanes 1–3. Molecular mass markers are shown on the right of each panel. B, integrity of BCL-3 ankyrin repeat domain is important for binding with B3BP. Structures of BCL-3 and its deletion mutants used in the experiment are shown. The black box represents the ankyrin repeat domain, which contains seven ankyrin-like units. BCL-3ΔC has the entire ankyrin repeat domain but lacks the C-terminal region. BCL-3Δank lacks the entire sixth and a part of the fifth and seventh ankyrin repeat. Equal volumes of reticulocyte lysate containing $^{35}$S-labeled products were run on an SDS-polyacrylamide gel and visualized by autoradiography (lanes 1–4, input). Lane 1, BCL-3; lane 2, BCL-3ΔC; lane 3, BCL-3Δank; lane 4, NFkB1. Equal amounts of reticulocyte lysate and purified GST (lanes 5, 7, 9, and 11) or GST-B3BP(N) (lanes 6, 8, 10, and 12) were mixed, and the proteins precipitated with glutathione-Sepharose were analyzed (lanes 5–12, GST pulldown). Exposure was as in A. The molecular mass markers are shown on the right of each panel.
Specifically associates with Nedd4 in vitro and in vivo and was ubiquitinated by Nedd4 in vitro. There has been no biochemical characterization of flN4BP2 so far; therefore we refer to it as BCL-3-binding protein in this paper. Salient features of B3BP include a consensus nucleotide-binding site, the Walker A motif, at residues 447–454, GLPGSGKS. Homology searches using the BLAST algorithm indicate that the nucleotide binding motif and its neighboring sequence most resemble those of 2/H11032,3/H11032-cyclic nucleotide 3/H11032-phosphodiesterase (CNP) of mammals and polynucleotide kinase (PNK) of bacteriophage T4 (PNK domain; Fig. 1, A and B), both of which possess 5'-polynucleotide kinase activity. The other motif shown by the analysis is the Smr domain found in the C-terminal region of B3BP (Fig. 1, A and C). The Smr domain has been described as a highly conserved sequence in the C-terminal region of the bacterial MutS2 family and phylogenetically speculated to be involved in DNA mismatch repair and meiotic chromosome crossing-over (30, 31). We also isolated a mouse homolog of B3BP cDNA that encodes a protein 70% identical to the human protein (data not shown). Notably, the N- and C-terminal regions of 300 amino acids containing the PNK and Smr domains, respectively, are more than 88% identical, suggesting functional conservation of these domains. Furthermore, in a survey of the Drosophila melanogaster genome data base, we found a putative ORF, LD21293, that encodes a polypeptide containing both the PNK and Smr domains at its N and C termini, respectively. However, the region between these domains exhibits no significant similarity. There is no ORF that encodes a single polypeptide containing these domains in the C. elegans or Saccharomyces cerevisiae genome, although stand-alone ORFs that encode the Smr domain are found in many lineages of eukaryotic and prokaryotic genomes (30, 31).

**B3BP Interacts with BCL-3 in Vitro**—The interaction between B3BP and BCL-3 was confirmed in vitro. GST fusion proteins containing the N-terminal (amino acids 2–631; GST-B3BP(N)) or C-terminal (amino acids 1171–1770; GST-B3BP(C)) regions of B3BP were expressed in E. coli and affinity-purified on glutathione-Sepharose beads. These proteins were then incubated with 35S-labeled BCL-3 translated in rabbit reticulocyte lysate. After extensive washing, the bound proteins were separated on an SDS-polyacrylamide gel and visualized by autoradiography. As shown in Fig. 2A, BCL-3 bound to the N-terminal portion but not the C-terminal portion of B3BP (lanes 5 and 6). In a converse experiment, GST-BCL-3 fusion protein bound to full-length B3BP (lane 7). The co-precipitation of BCL-3 and GST-B3BP is due to homophilic interaction (37, 38). We used luciferase, which is unrelated to BCL-3 or B3BP, as a negative control and found that it bound neither to GST nor to GST fusion proteins. It has been shown that the ankyrin repeat domain of BCL-3 is important for the interaction of the homodimer of the NFKB1 p50 subunit (38). Therefore, we next examined the involvement of the ankyrin repeat domain in the association with B3BP (Fig. 2B). Wild type BCL-3 and the C-terminal deletion mutant, BCL-3(ΔC), which lacks the C-terminal portion (amino acids 353–437) but retains the entire ankyrin repeat domain, specifically associated with GST-B3BP(N) (lanes 5–8). However, another mutant BCL-3(Δank), which lacks a part of the ankyrin repeat (amino acids 280–339) did not bind to GST-B3BP(N) (lanes 9 and 10), indicating the importance of the ankyrin repeat domain. BCL-3(Δank) failed to interact with the NFKB1 p50 subunit as well (data not shown). Although the ankyrin repeat domains of BCL-3 and NFKB1 and NFKB2 are homol-
and HA-tagged proteins were expressed in 293T cells by transient co-transfection (Fig. 3). Whole cell extracts were prepared after 36 h of transfection and mixed with anti-FLAG antibody-conjugated resin. After extensive washing, the bound proteins were eluted in 1× Laemmli’s sample buffer and subjected to Western blot analysis using anti-HA antibody. We found that FLAG-tagged full-length B3BP interacted with not only HA-tagged BCL-3 (lane 8) but also p300 (lane 6) in 293T cells. Moreover, we observed homophilic interaction with B3BP in 293T cells (lane 7). A converse experiment showed that FLAG-tagged BCL-3 interacts with HA-tagged full-length B3BP (lane 11) but not with p300 (lane 10). FLAG-tagged CBP interacted with HA-tagged B3BP (lane 15) but not with BCL-3 (lane 16), a similar result as p300.

**B3BP Binds and Hydrolyzes ATP**—Because B3BP possesses a domain homologous to PNK, which binds to ATP and hydrolyzes it, we investigated these activities for B3BP. Expression vectors encoding FLAG-tagged B3BP (B3BP-FLAG) or its derivatives, B3BP(K/A)-FLAG and B3BP(K/R)-FLAG in which the Lys residue within the ATP-binding motif was substituted with Ala and Arg, respectively, were introduced into 293T cells, and whole cell lysate was prepared at 36 h after transfection. FLAG-tagged proteins were purified using the anti-FLAG antibody-immobilized beads and incubated with [α-32P]8-azido-ATP. After cross-linking by UV irradiation followed by extensive washing to remove unbound ATP, the samples were eluted and separated on SDS-polyacrylamide gel and visualized by CBB staining and autoradiography. Fig. 4A clearly shows that wild type B3BP has the ability to bind ATP and that B3BP(K/A) and B3BP(K/R) exhibit reduced ATP binding, particularly the K/A mutant. Next we examined ATP-hydrolisis by B3BP. FLAG-tagged B3BP and B3BP(K/R) were purified and cross-linked with [γ-32P]8-azido-ATP. After extensive washing, the samples were incubated at 30 °C in the presence or absence of yeast transfer RNA (ytRNA) and subjected to SDS-PAGE analysis as above. CBB staining of the gel shows that the amount of B3BP and B3BP(K/R) did not change after the incubation (Fig. 4B), and it was confirmed by the densitometric quantification of the gel. However, the associated 32P at position γ of the ATP cross-linked to B3BP but not to B3BP(K/R) decreased with incubation. Interestingly, the decrease was accelerated in the presence of ytRNA (lane 3). These results indicate that B3BP has binding and hydrolyzing activity for ATP that is augmented in the presence of ytRNA. As with other ATPases, the critical Lys (Lys453) residue is important for these activities (39, 40).

**ATP Binding Activity of B3BP Is Required for the Interaction with BCL-3 and p300**—Because the PNK domain coincides with the region required for association with BCL-3 and p300, we examined the involvement of ATP binding activity in the protein-protein interaction. FLAG-tagged B3BP, B3BP(K/A), or B3BP(K/R) was co-expressed with HA-tagged BCL-3 or p300 and analyzed as in Fig. 3 (Fig. 5A). The association of B3BP with BCL-3 was impaired by the substitution of Lys453 with Ala or Arg, the former resulting in a barely detectable association (lanes 6–8). Interestingly, the addition of 2 mM ATP to the binding mixture markedly enhanced the association between B3BP and BCL-3 in vitro (Fig. 5B). For the association with p300, Lys453 appears to be indispensable because the association of p300 with either the K/A or K/R mutant was virtually absent (lanes 10–12). On the other hand, the mutations of Lys453 exhibited little effect on the homophilic interaction of B3BP (lanes 14–16).

**B3BP Possesses 5′-Polynucleotide Kinase Activity**—Primary sequence alignment of the ATP-binding motif showed a high level of conservation between B3BP and PNK. We investigated the PNK activity of B3BP. First, the PNK domain of B3BP (amino

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**Fig. 4. ATP-binding and hydrolyzing activity of B3BP. A, ATP binding activity of B3BP.** FLAG-tagged B3BP and its derivatives, B3BP(K/A) and B3BP(K/R), were transiently expressed in 293T cells and adsorbed to anti-FLAG matrix. An ATP binding assay using [α-32P]8-azido-ATP was conducted as described under “Experimental Procedures,” and the proteins were run on an SDS-polyacrylamide gel and visualized by autoradiography and CBB staining. **B, ATP-hydrolyzing activity of B3BP.** FLAG-tagged B3BP (lanes 1–6) and B3BP(K/R) (lanes 46) were subjected to UV cross-linking with [γ-32P]8-azido-ATP and incubated (30 min; lanes 2, 3, 5, and 6) or not (0 min; lanes 1 and 4) in the presence (lanes 3 and 6) or absence (lanes 1, 2, 4, and 5) of yeast transfer RNA (ytRNA) (“Experimental Procedures”). The proteins were separated by SDS-PAGE, stained with CBB, and then autoradiographed. Radioactivities cross-linked with B3BP were quantitated and normalized relative to the protein amount determined by densitometric analysis of the CBB staining (bottom panel). We repeated the experiment and reproduced essentially the same results.
acids 394–630) fused to GST (GST-B3BP(M)) was produced in 
E. coli and purified along with the control GST. GST-
B3BP(M) but not GST efficiently phosphorylated both 40-bp 
double-stranded DNA with blunt ends and 40-base single-
stranded DNA (Fig. 6A, lanes 4 and 6). Moreover, GST-
B3BP(M) phosphorylated yeast transfer RNA, although less 
efficiently (lane 8). The phosphorylation of DNA substrate 
with T4 PNK using cold ATP prior to the assay totally inhib-
ited the incorporation of \(^{32}\)P into the substrates by GST-
B3BP(M), indicating that B3BP phosphorylates the 5’ hydroxyl group of the substrate (data not shown). Next we 
examined full-length B3BP produced in mammalian cells for 
the PNK activity. FLAG-tagged B3BP and its derivatives, 
B3BP(K/A) and B3BP(K/R), were expressed in 293T cells and 
purified using anti-FLAG antibody. The recombinant pro-
teins were subjected to the in vitro PNK assay (Fig. 6B). 
Full-length B3BP phosphorylated the polynucleotide sub-
strates efficiently (lanes 2, 6, and 10); however, the activity of 
B3BP(K/A) and B3BP(K/R) was below the detectable level 
(lanes 3, 4, 7, 8, 11, and 12). Thus, B3BP has intrinsic 5’ PNK 
activity for both DNA and RNA.

Smr Domain of B3BP Shows Nicking Endonuclease Activ-
ity—The Smr domain, which is found in many eukaryotes, is 
proposed to act as a nicking endonuclease and participate in 
gene recombination (30). Therefore, we investigated the nick-
ing endonuclease activity of the Smr domain. The Smr domain 
of B3BP was expressed in E. coli as GST fusion protein and 
purified. After the removal of the GST moiety by protease 
treatment, the Smr domain was further purified by ion-ex-
change chromatography. The purified GST or Smr domain was 
incubated with supercoiled circular plasmid DNA, and the 
samples were separated by agarose gel electrophoresis. Fig. 7 
shows that the Smr domain but not GST converted supercoiled 
DNA into a nicked open circular form in a dose-dependent 
manner, and no linear species was detected under these con-
ditions. Furthermore, linearized plasmid was not detectably 
affected by incubation with Smr, suggesting that it does not 
have exonuclease activity (data not shown). These results in-
dicate, for the first time, that the Smr domain of B3BP has 
nicking endonuclease activity but no significant double strand 
cleavage or exonuclease activity.
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Fig. 6. Polynucleotide kinase activity of B3BP. A, polynucleotide kinase activity of ATP-binding domain of B3BP. Protein profiles of the purified GST (lane 1) and GST-B3BP(M) (lane 2) are shown by CBB staining. Lane M, size marker. 10 pmol of GST (lanes 3, 5, and 7) and GST-B3BP(M) (lanes 4, 6, and 8) were subjected to polynucleotide kinase assay as described under “Experimental Procedures” using 100 pmol of 5'-OH ends of single-stranded DNA (ssDNA, lanes 3 and 4) or double-stranded DNA (dsDNA, lanes 5 and 6) and 2.5 μg of tRNA (lanes 7 and 8) as a substrate. The polynucleotides were run on a denaturing 10% polyacrylamide gel and subjected to autoradiography. B, polynucleotide kinase activity of FLAG-tagged B3BP. FLAG-tagged B3BP and its derivatives, B3BP(K/A) and B3BP(K/R), were expressed in 293T cells and purified by anti-FLAG agarose matrix. Polynucleotide kinase assay was performed as shown in A except the proteins remained bound to the matrix during the assay. The polynucleotide substrates were analyzed on a denaturing 10% polyacrylamide gel and subjected to autoradiography as shown in the upper panel. The bound proteins were analyzed on a NuPAGE 4–12% Bis-Tris gel and by CBB staining as shown in the lower panel. The molecular mass markers are shown on the left of the panel. Arrow, B3BP and the mutants. Asterisk and dot, anti-FLAG Ig heavy and light chain, respectively.

DISCUSSION

In this study we identified B3BP, which associates specifically with BCL-3, from a yeast two-hybrid screen of a human spleen cDNA library and characterized it. A previous report has described the isolation of factors interacting with BCL-3 by yeast two-hybrid screening (19). However, only NFKB1 was found in our study, probably because of the different screening methodology used, e.g. a different bait (full-length versus ankyrin domain alone) or a different cDNA library.

The amino acid sequence of B3BP revealed the presence of the PNK and Smr domains near the N- and C-terminal region, respectively. Indeed, we demonstrated that B3BP is a PNK (Fig. 6). The biological function of mammalian CNP still remains unclear; however, T4 PNK functions in an RNA repair pathway in collaboration with a phage-encoded RNA ligase (41). T4 PNK is the founding member of the family of bifunctional 5'-kinase/3'-phosphatase enzymes that heal broken termini in RNA or DNA by converting 3'-PO4/5'-OH ends into 3'-OH/5'-PO4 ends, which are then feasible for sealing by RNA or DNA ligases (40). However, B3BP does not show any homology to the 3'-phosphatase domain of the T4 PNK family. In fact we failed to detect the 3'-phosphatase activity of full-length B3BP using an oligonucleotide with 3'-PO4 ends as substrate (data not shown). The biological function of 5'-kinase free of 3'-phosphatase activity is totally unknown at present; however, it is of note that 5'-phosphate is required for small interfering RNA to function in the RNA interference pathway (42). A functional ATP-binding motif is required for B3BP to associate with BCL-3 and CBP/p300 (Fig. 5); notably the presence of ATP enhanced the former association. These results suggest that the assembly of the complex containing B3BP and its enzymatic activity are coordinately regulated.

A BLAST survey of the genome data base indicates that B3BP could be the only ORF encoding the Smr domain in humans. Although the biological function of the Smr domain is totally unknown, the domain is speculated to have a role in mismatch repair or meiotic chromosome crossing-over and expected to function as a nicking endonuclease (30). Indeed, we found that the Smr domain of B3BP showed nicking endonuclease activity (Fig. 7). Such activity is required for various kinds of biological processes, such as the retrotransposition of self-splicing introns, the excision repair of damaged DNA, and replication-coupled mismatch repair. It is worth noting that there is no gene for an eukaryotic homolog of MutH, the nicking endonuclease required for mismatch repair in E. coli (30). It is also reported that some of the mismatch repair proteins are involved in transcription-coupled excision repair of DNA (43).
Recently it was reported that p300/CBP associates with proliferating cell nuclear antigen and thymine DNA glycosylase, suggesting a functional coupling of transcriptional activation to DNA repair synthesis and base mismatch repair, respectively (44, 45). Therefore, investigations of the Smr domain and B3BP might help us to understand the mechanisms underlying eukaryotic mismatch repair and its relation to transcriptional activation. Nicking endonuclease activity is also supposed to be required for the recombination of the Ig gene in germinal center B cells, i.e. class switch recombination and somatic hypermutation, which are coupled with transcriptional activation of the intron switch region and Ig gene itself, respectively (27). Moreover, studies with gene targeted mice demonstrated that mismatch repair factors are involved in these processes (28).

All of the findings observed suggest that B3BP is involved in DNA or RNA transaction processes, e.g. transcription-coupled DNA repair or genetic recombination, because (i) B3BP interacts with not only BCL-3 but also p300/CBP, both of which are involved in gene activation; (ii) B3BP shows 5'-polyadenylate kinase activity, which is required for DNA or RNA repair by converting 5'-OH broken termini into ligation-competent 5'-PO₄ ends; and (iii) the C-terminal region of B3BP contains the Smr domain, which is phylogenetically speculated to have a role in mismatch repair or meiotic recombination and indeed shows nicking endonuclease activity.

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