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Poly (ADP-Ribose) Polymerase-1 Binds to BCL2 Major Breakpoint Region and Regulates BCL2 Expression

Nan Yang,[1,2] Feiran Gong,[1] Luan Sun,[1] Dapeng Yang,[2] Xiao Han,[1] Changyan Ma,[1,2*] and Yujie Sun[1,2,3*]

[1]*Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University, Hanzhong Rd. #140, Nanjing, P.R. China, 210029*

[2]*Department of Cell Biology and Medical Genetics, Nanjing Medical University, Hanzhong Rd. #140, Nanjing, P.R. China, 210029*

[3]*Cancer Center, Nanjing Medical University, Hanzhong Rd. #140, Nanjing, P.R. China, 210029*

ABSTRACT

BCL2, originally identified as a proto-oncogene in B-cell lymphoma, is a key regulator of apoptosis. Although it is more than 200 kb in length, at least 70% of the t(14;18) translocation in follicular lymphomas occurs at the BCL2 major breakpoint region (mbr), located in the 3′-untranslated region (3'-UTR). We have previously found that the mbr is a regulatory element which positively regulates BCL2 expression and this regulatory function was closely associated with SATB1, which binds to a 37 bp mbr (37 mbr) in the 3′-end of the mbr directly. However, the precise molecular mechanisms by which the mbr regulates gene expression are not fully understood. In this study, we purified Poly(ADP-ribose) polymerase-1 (PARP-1) from the DNA–protein complexes formed by 37 mbr in Jurkat cells and demonstrated that PARP-1 participates in the 37 mbr–protein complex's formation in vitro and in vivo. Functional analysis showed that overexpression of PARP-1 decreases 37 mbr regulatory function and BCL2 expression. Conversely, knockdown of PARP-1 with RNAi increases BCL2 expression. Taken together, the present findings indicate that PARP-1 is a component of BCL2 37 mbr–protein complexes, and PARP-1 is involved in the regulation of BCL2 expression. These findings are helpful in understanding the regulatory mechanisms of BCL2 expression. J. Cell. Biochem. 110: 1208–1218, 2010.

KEY WORDS: BCL2; POLY(ADP-RIBOSE) POLYMERASE-1; 37 MBR; REGULATION; SATB1

BCL2 was first cloned in cells of human follicular B-cell lymphoma with the t(14;18) chromosomal translocation [Tsujimoto et al., 1985a,b]. It was originally identified as a proto-oncogene involved in cellular growth, differentiation, and cell cycle regulation [Hockenbery et al., 1990; Sato et al., 1994; Chen et al., 1997; Deng et al., 2003]. Overexpression of BCL2 blocks apoptosis induced by DNA damage and gene mutations, and promotes the initiation and progression of tumors.

The t(14;18)(q32;q21) chromosomal translocation is a characteristic of follicular lymphoma and a frequent abnormality in other types of non-Hodgkin lymphoma. This event constitutively activates the BCL2 gene and disrupts control of apoptosis [Rabkin et al., 2008]. Although the BCL2 gene is more than 200 kb in length, at least 70% of the translocation in follicular lymphomas occurs at the BCL2 major breakpoint region (mbr), located in the 3'-untranslated region (3'-UTR) of BCL2 [Cleary and Sklar, 1985; Tsujimoto et al., 1985a,b]. Our previous investigations have demonstrated that the mbr positively regulates BCL2 gene expression, and deletion of the mbr significantly decreases the transcriptional activity of the corresponding allele in the mbr^{+}/mbr^{-} Nalm-6 cells. In addition, the BCL2 allele with the mbr deletion has a slower response to apoptotic stimuli than the wild-type allele [Zhang et al., 2006; Ma et al., 2007]. However, to date, the precise molecular mechanisms by which the mbr regulates BCL2 gene expression are not fully understood.

Special AT-rich sequence-binding protein 1 (SATB1), a matrix attachment region (MAR)-binding protein, binds at 37 mbr, which is an AT-rich region located in the 3'-end of the BCL2 mbr

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*Correspondence to: Changyan Ma and Yujie Sun, Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University, Hanzhong Rd. #140, Nanjing, P.R. China, 210029.
E-mail: cyma@njmu.edu.cn; yujiesun@njmu.edu.cn

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[Ramakrishnan et al., 2000]. As a class of transcriptional regulators that function as a landing platform for some chromatin remodeling enzymes and transcription factors, SATB1 regulates large chromatin domains by providing sites for organization of DNA sequences and regulating region-specific histone modification [Yu et al., 2002]. It has been suggested that the regulatory function of the mbr depends on the formation of diverse protein complexes, and SATB1 is not the sole DNA-binding protein that binds to 37 mbr [Ramakrishnan et al., 2000; Ma et al., 2007].

Poly(ADP-ribose) polymerase-1 (PARP-1) is the founder member of the PARP family, which contains as many as 18 distinct proteins in humans [Ame et al., 2004]. It has a highly conserved structural and functional organization including (1) an N-terminal double zinc finger DNA-binding domain (DBD), (2) a nuclear localization signal, (3) a central automodification domain, and (4) a C-terminal catalytic domain [D'Amours et al., 1999; Kraus and Lis, 2003]. It synthesizes ADP-ribose on various nuclear protein acceptors with nicotinamide adenine dinucleotide (NAD^+), resulting in linear or branched polymers of PAR up to 200 U [D'Amours et al., 1999]. Poly(ADP-ribosyl)ation has been implicated in many cellular processes including DNA replication, transcription, and the maintenance of genomic stability. The roles of PARP-1 in transcriptional regulation have been demonstrated in several physiological contexts, and two different mechanisms have been proposed: (1) modulating chromatin structure, and (2) functioning as part of enhancer/promoter binding complexes or insulators in conjunction with other DNA binding factors and coactivators [Kraus and Lis, 2003; Kraus, 2008]. In the present study, we purified PARP-1 as a 37 mbr binding protein using streptavidin magnetic particles and demonstrated that PARP-1 binds to 37 mbr by electrophoretic gel mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). In addition, we studied the effects of PARP-1 on 37 mbr regulatory function and BCL2 expression.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

Human T lymphoid cell line Jurkat and human pre-B lymphoid cell line Nalm-6 are generous gifts from Dr. Krontiris' Laboratory at City of Hope National Medical Center (Los Angeles). Human breast cancer cell line MCF-7 and human embryonic kidney cell line HEK 293 were purchased from American Type Culture Collection (Rockville, MD). Jurkat cells and Nalm-6 cells were cultured at densities between 0.2 and 1.0×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 100 U/ml penicillin/streptomycin, and 1 mM sodium pyruvate. MCF-7 and HEK 293 cells were grown in MEM (MCF-7) and DMEM (HEK 293) medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and 100 U/ml penicillin/streptomycin. The cells were incubated at 37°C in humidified atmosphere containing 95% air/5% CO_2.

PLASMIDS

The expression plasmid pPARP31 containing human PARP-1 is a generous gift from Dr. Alexander Bürkle at DKFZ of Heidelberg, Germany. The control plasmid pL15TK was prepared from pPARP31. Luciferase reporter plasmid 37 mbr-Luc containing 37 mbr was constructed by inserting the 37 mbr sequence into the pGL3-promoter vector, 279 mbr-Luc and 242 mbr-Luc were constructed as previously described [Zhang et al., 2006]. All of the plasmids were confirmed by sequencing at Invitrogen Corporation (Shanghai, China). The miRNA plasmid SR75-1 and the control plasmid SR(−) were purchased from Invitrogen Corporation.

PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagents and Halt™ Protease Inhibitor Cocktail Kit (Pierce Biotechnology, Inc.), as recommended by the manufacturer. Briefly, Jurkat cells were harvested and washed with cold PBS. The cell pellet was then incubated with CER I buffer for 10 min. After incubation with the CER II detergent solution for 1 min, preparations were centrifuged at 16,000*g* for 5 min. The nuclear pellet was resuspended in nuclear extraction buffer (NER) and vortexed four times. After centrifugation at 16,000*g* for 10 min, the supernatant (nuclear extract) was collected, aliquoted, and frozen at −80°C until used. All the centrifugation steps were performed at 4°C, and all buffers were chilled on ice.

IMMUNOMAGNETIC BEAD ISOLATION OF DNA-BINDING PROTEIN

The following repeat sequences of 37 mbr for self-primed PCR were synthesized: 37 mbr R–F (5′-TATGAAAGGTTTACATTGTCAAAGT-GATGAATATGGATATGAAAGGTTTACATTGTCAAAGTGATGAAT-ATGGA-3′) and 37 mbr R–R (5′-TCCATATTCATCACTTTGACAATG-TAAACCTTTCATATCCATATTCATCACTTTGACAATGTAAACCTTT-CATA-3′), with the oligonucleotides phosphorylated at the 5′-end. The concatameric oligonucleotides containing the 37 mbr sequence were prepared by self-primed PCR with the primers 37 mbr R–F and 37 mbr R–R. The PCR mixture (100 μl) contained 20 pmol/μl of each primer, 0.2 mM of each deoxynucleotide, 4 mM $MgSO_4$, 1× Pwo polymerase buffer, and 2.5 U Pwo DNA polymerase (Roche Applied Science, Mannheim, Germany). PCR conditions consisted of 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 45 s, and final elongation at 72°C for 7 min. The concatameric products were ligated to the streptavidin magnetic particles (Roche Applied Science), and incubated with nuclear extracts (50 μg protein) of Jurkat cells as recommended by the supplier. The bound nuclear proteins were washed and then eluted with an increasing linear gradient (0.4, 0.6, 1.0 M) of KCl according to the manufacturer's instructions. Fractions were run on a 10% SDS–PAGE, and visualized using the SilverQuest Silver Staining Kit (Invitrogen, Paisley, UK).

MALDI-TOF MS AND MS/MS

The protein bands were excised from the gel, digested with trypsin, and subjected to MALDI-TOF MS for peptide mass fingerprint and MS/MS for peptide sequencing. The data obtained were screened against the SWISS-PROT database using the MASCOT search engine. The searches were carried out with a peptide mass accuracy tolerance of 100 ppm for external calibration. The search criteria used were as follows: one missed cleavage per peptide and variable modifications of carbamidomethyl (C).

ELECTROPHORETIC GEL MOBILITY SHIFT ASSAY

EMSA were performed using the gel shift assay kit (Promega Corp., Madison) according to the instructions of the manufacturer. Oligonucleotide probes were annealed and labeled with [γ-^{32}P] ATP and T4-polynucleotide kinase (New England Biolabs Ltd., Beijing, China). Nuclear extracts (10 μg) of Jurkat cells were incubated with 0.05 pmol of the ^{32}P-labeled probe in a total volume of 10 μl as recommended by the manufacturer. The protein–DNA complexes were separated on a 5% PAGE. Signals were recorded on X-ray film. The sequences of the 37 mbr probe and the non-specific competitive probe were as follows: 37 mbr-F (5′-TATGAAAGGTT-TACATTGTCAAAGTGATGAATATGGA-3′); 37 mbr-R (5′-TCCA-TATTCATCACTTTGACAATGTAAACCTTTCATA-3′); SP1-F (5′-ATTCGATCGGGGCGGGGCGAGC-3′); and SP1-R (5′-GCTCGCCCCGCCCCGATCGAAT-3′). For competition experiments, either specific or non-specific oligonucleotide competitor was added to the binding mixture 10 min before addition of the labeled probe. For supershift experiments, either anti-PARP-1 antibody (Cell Signalling Technology, Danvers, MA) or polyclonal anti-SATB1 serum were added during preincubation.

CHROMATIN IMMUNOPRECIPITATION

ChIP assay was carried out using the ChIP assay kit (Upstate/Chemicon, Temecula, CA) as per the manufacturer's instructions. Jurkat cells (2×10^7) were harvested and fixed in 1% (v/v) formaldehyde in minimal culture medium for 10 min at room temperature. After washing with cold PBS, cells were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1). The lysates were sonicated to shear the genomic DNA to 200–1,000 bp in size using Sonicator VCX130 (Sonics & Materials, Inc., Newtown). Chromatin solution was precleared with protein A-agarose beads for 30 min at 4°C, and the supernatant was collected. One percent of the chromatin solution was reserved as input sample. The remaining chromatin was incubated overnight at 4°C with anti-PARP-1 antibody, and IgG as a negative control. The chromatin/antibody complexes captured on the beads were extensively washed and then eluted with 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$). The immunoprecipitated and 1% of the chromatin solution were incubated for 4 h at 65°C to reverse the cross-links. After treatment with proteinase K and RNase for 1 h at 37°C, the reaction was stopped and the immunoprecipitated DNA was then amplified by PCR using primers corresponding to the mbr of BCL2. Sequences of the primers used were: forward primer (5′-GGGCCTCAGGGAACA-GAA-3′) and reverse primer (5′-TAG CAGCACAGGATTGGA-3′). The PCR cycling parameters were as follows: 95°C, 4 min; 32 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s. An aliquot of input sample was amplified by PCR along with aliquots of immunoprecipitated DNA to assess the relative binding of PARP-1. The PCR products were subjected to gel electrophoresis, stained with ethidium bromide, and analyzed on Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA).

CELL TRANSIENT TRANSFECTION

Jurkat and Nalm-6 cells were transfected using an electroporator (Bio-Rad) at 975 μF and 250 V in a 0.4-cm cuvette at a concentration of 1×10^7 cells/350 μl in RPMI 1640 medium containing 10% FCS.

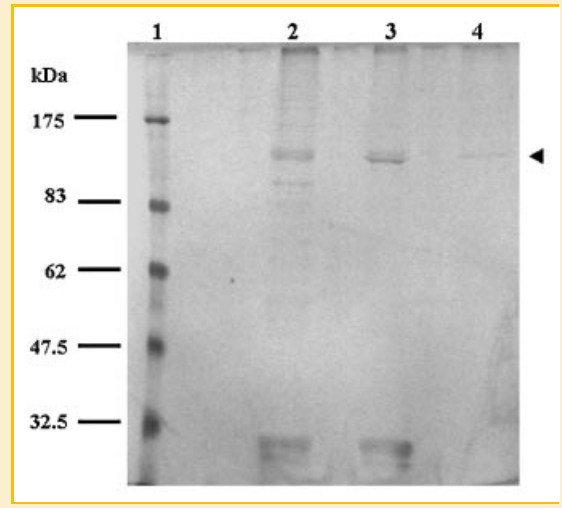

Fig. 1. Isolation of proteins binding to the 37 mbr. Nuclear extracts (50 μg total protein) were prepared from Jurkat cells, and mixed with streptavidin magnetic particles ligated with concatameric oligonucleotides of the 37 mbr sequence. The magnetic particles were washed and eluates were collected. Fractions were run on a 10% SDS–PAGE and visualized by silver staining. A protein of 113 kDa was eluted (black arrow). Lane 1, protein marker; lanes 2–4, eluates with 0.4, 0.6, and 1.0 M KCl, respectively. This experiment was carried out three times.

Each electroporation was plated into a 60-mm diameter tissue culture dish and incubated under normal conditions. MCF-7 cells were transfected using Lipofectamine™ 2000 reagent (Invitrogen Corp.) according to the manufacturer's instructions, and cells were plated on 6- or 12-well plates to achieve 90–95% confluence for transfection on the following day. HEK 293 cells were transfected

TABLE I. MALDI-TOF MS Results of PARP-1

PARP-1	Amino acid residues	*m/z*
34-46	MAIMVQSPMFDGK	1454.7544
65-77	**HPDVEVDGFSELR**	**1499.7617**
108-118	TLGDFAAEYAK	1185.6406
142-155	MVDPEKPQLGMIDR	1628.8569
156-164	WYHPGCFVK	1193.6178
167-181	EELGFRPEYSASQLK	1753.9079
262-268	ELLIFNK	876.5315
269-281	QQVPSGESAILDR	1399.7672
305-319	SDAYYCTGDVTAWTK	1737.8351
452-466	VVSEDFLQDVSASTK	1624.8612
486-495	**AEPVEVVAPR**	**1066.6730**
528-547	GGAAVDPDSGLEHSAHVLEK	1988.9831
551-563	VFSATLGLVDIVK	1361.8507
582-586	YWIFR	784.4056
621-628	TGNAWHSK	900.4776
664-673	LPKPVQDLIK	1150.7669
684-694	AMVEYEIDLQK	1338.7048
704-734	QIQAAYSILSEVQQAVSQGSSDSQILDLSNR	3335.6859
735-746	FYTLIPHDFGMK	1468.7821
747-760	KPPLLNNADSVQAK	1494.8729
761-778	VEMLDNLLDIEVAYSLLR	2106.1064
806-814	DSEEAEIIR	1061.5629
819-837	NTHATTHNAYDLEVIDIFK	2202.0902
841-846	EGECQR	778.2937
858-864	LLWHGSR	868.4854
865-877	**TTNFAGILSQGLR**	**1377.7951**
878-892	IAPPEAPVTGYMFGK	1577.8611
893-902	GIYFADMVSK	1130.6006

Peptides in bold were sequenced with MS/MS.

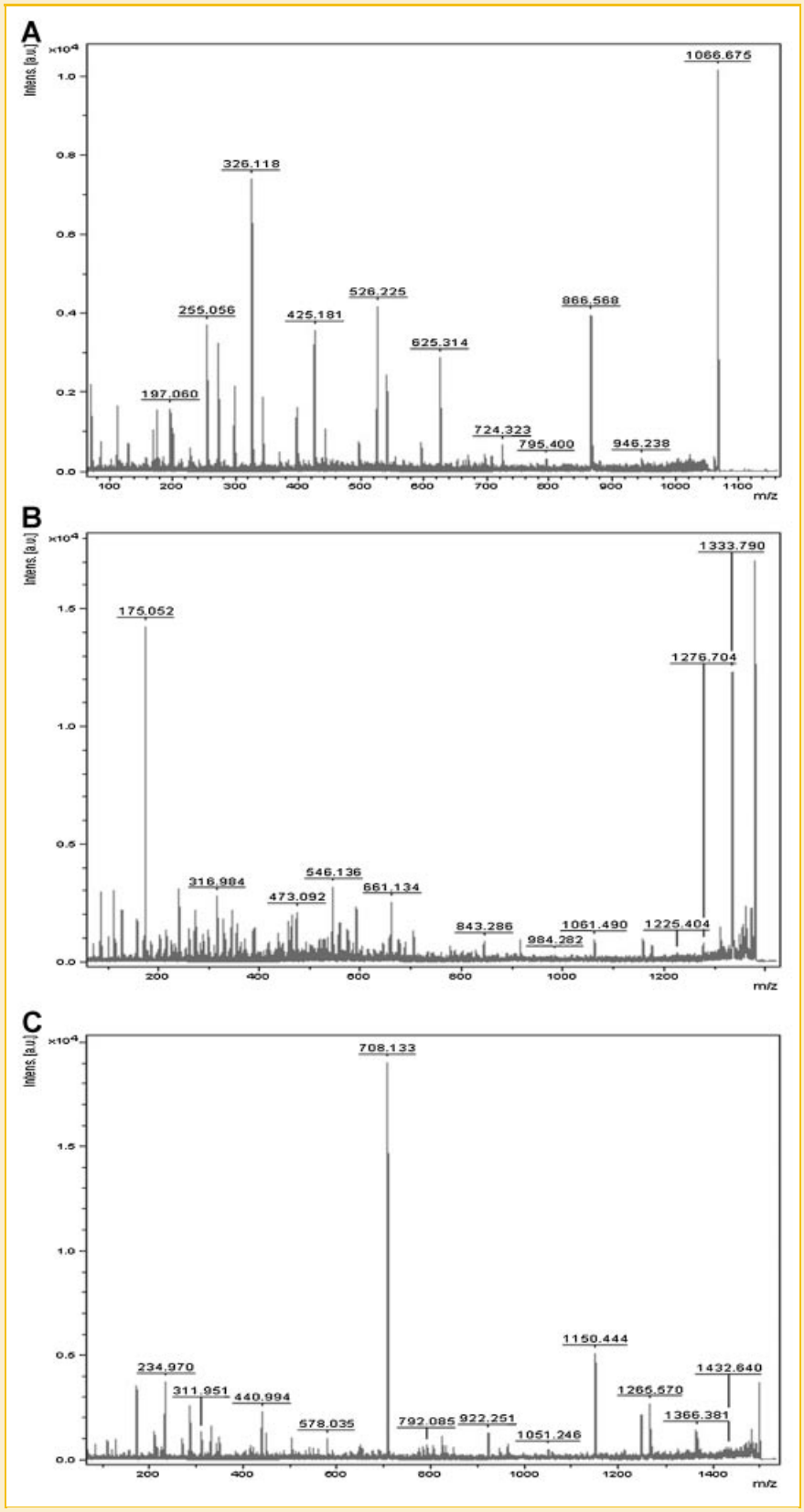

Fig. 2. PARP-1 was identified by peptide mass fingerprint and peptide sequencing. (A–C) MALDI-TOF MS/MS spectra of each parent peptide ion m/z 1066.6730, 1377.7950, and 1499.7617 in Table I are (A), (B), and (C), respectively.

using Calcium Phosphate Cell Transfection reagent (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions, and cells were plated on 6- or 12-well plates to achieve 80% confluence for transfection on the following day.

LUCIFERASE ASSAY

Jurkat, Nalm-6, MCF-7, or HEK 293 cells were transfected with 37 mbr-luciferase/279 mbr-luciferase/242 mbr-luciferase reporter plasmid, β-galactosidase plasmid, and PARP-1 expression plasmid pPARP31/empty plasmid pL15TK, respectively. Forty-eight hours after transfection, cells were washed with PBS and lysed with 1× Reporter lysis buffer. Twenty microliters of cell lysates were assayed for luciferase activity using Luciferase assay reagent (Promega Corp.). β-Galactosidase activity was measured to normalize the luciferase activity.

WESTERN BLOT

Cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 0.5% NP-40, and 0.01% SDS) containing protease inhibitors. Total protein (20 μg) was boiled for 5 min in 1× loading buffer, chilled on ice, and then separated on 10% SDS–PAGE. Following transfer onto PVDF membranes (Bio-Rad), non-specific protein interactions were blocked by incubation in 5% non-fat dry milk in TST-buffer (50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20 pH 7.6) at room temperature for 1 h. Membranes were then incubated overnight at 4°C with polyclonal anti-PARP-1 antibody (Cell Signalling Technology), anti-BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actin monoclonal antibody (Sigma Chemical Co., St Louis, MO) in fresh blocking buffer. Unbound antibody was removed by three 10 min washes in TST buffer. Membranes were then incubated with horseradish peroxide-conjugated individual secondary antibody (R&D systems Inc., Minneapolis) for 1 h at room temperature, followed by three 10 min washes with TST buffer. The blot was developed with ECL reagent (GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK). Prestained markers were used as molecular weight standards.

RNA ISOLATION AND REAL-TIME PCR ANALYSIS

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad) according to the manufacturer's protocol. cDNAs were synthesized from total RNA (1 μg) using random primers and ReverTra Ace-α-™ First Strand cDNA Synthesis Kit (TOYOBO Co., Ltd., Osaka, Japan). Real-time PCR was performed with ABI Prism® 7000 Real-Time PCR System (Applied Biosystems, Foster City). The primers of BCL2 were as follows: BCL2NRT-F (5′-TCGCCCTGTGGATGACTGAG-3′) and BCL2NRT-R (5′-CAGAGTCTTCAGAGACAGCCAGGA-3′). β-actin was amplified simultaneously as an internal control. The reaction (20 μl) contained the cDNA from above, forward and reverse primers, and SYBR GREEN PCR Master Mix (TOYOBO, Co., Ltd.). Amplification conditions were 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 63°C for 1 min, and extension at 72°C for 30 s.

RESULTS

PURIFICATION AND IDENTIFICATION OF 37 MBR-BINDING PROTEINS FROM JURKAT NUCLEAR EXTRACTS

To identify the proteins participating in 37 mbr–protein complex's formation, we performed DNA affinity chromatography. Nuclear extracts from Jurkat cells were incubated with streptavidin magnetic particles ligated to concatameric oligonucleotides of the 37 mbr sequence, in the presence of excess poly(dI–dC) to compete with non-specific DNA-binding proteins. Nuclear proteins bound to concatameric oligonucleotides were then eluted and resolved by SDS–PAGE. Several bands were detected in the elutions (Fig. 1). The protein bands were then excised from the gel, and subjected to MALDI-TOF MS (Table I) and MS/MS (Fig. 2A–C). PARP-1 (Fig. 1, black arrow) was identified in 47 peptides with a highly significant probability score and with coverage of 46% of the protein from amino acid positions 34–902.

PARP-1 BINDS TO 37 MBR IN VITRO

To demonstrate the specific binding of PARP-1 to the 37 mbr, we performed an EMSA using cold (unlabeled) competitors and anti-PARP-1 antibody. Several bands were detected by gel electrophoresis and autoradiography after incubation of the radiolabeled

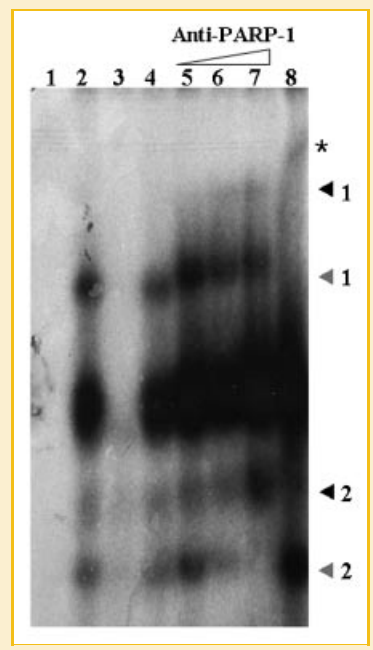

Fig. 3. Demonstration of PARP-1 as a 37 mbr-binding protein by Gel shift assays using anti-PARP-1 antibody. Lane 1 is a free probe control without protein lysate; lane 2 is no antibody and shows the 37 mbr–protein complexes from Jurkat nuclear extracts; lanes 3 and 4 show competition EMSA with the 37 mbr target. Lane 3 is with 5 pmol unlabeled 37 mbr sequence as a specific-competitor, lane 4 is with 5 pmol unlabeled SP1-binding sequence as a non-specific-competitor; lanes 5–7 are 1, 3, and 5 μl anti-PARP-1 antibody, respectively. Black arrows 1 and 2 indicate the supershifted protein–DNA complexes; gray arrows 1 and 2 indicate the corresponding protein–DNA complexes of supershifted bands; lane 8 is 1 μl anti-SATB1 antibody as a positive control, asterisk indicates the supershifted protein–DNA complex containing SATB1.

37 mbr oligonucleotide probe with the nuclear extracts of Jukart cells (Fig. 3, lane 2). In the competition assay with excess of unlabeled 37 mbr (cold probe), all the bands disappeared (Fig. 3, lane 3). In contrast, when unlabeled SP1-binding sequence (a CG-rich sequence) was used as a non-specific competitive probe, these bands still appeared (Fig. 3, lane 4). These results confirmed that the bands in lane 2 were 37 mbr-specific binding bands. Moreover, the addition of anti-PARP-1 antibody (1, 3, and 5 μl) formed two supershifted bands (Fig. 3, lanes 5, 6, 7, black arrows 1 and 2) and the density of the supershifted bands was increased in a dose-dependent manner with the increased amount of antibody, suggesting PARP-1 was involved in both complexes 1 and 2 (Fig. 3, gray arrows 1 and 2). When 1 μl of anti-SATB1 antibody was added as a positive control, a supershifted band was visualized as expected (Fig. 3, lane 8, asterisk). However, complex 1 which contains PARP-1 (Fig. 3, gray arrow 1) disappeared in the presence of anti-SATB1 antibody, suggesting that complex 1 may represent a large protein–DNA complex containing both PARP-1 and SATB1 proteins.

PARP-1 can bind DNA in a sequence-specific manner, and various binding sequences have been described [Akiyama et al., 2001; Nirodi et al., 2001; Zhang et al., 2002; Huang et al., 2004; Ambrose et al., 2007; Okada et al., 2008]. To determine which part of the 37 mbr is important for PARP-1 binding, we performed deletion analysis of the 37 mbr region (Fig. 4A). Complex 1 which contains PARP-1 (Fig. 3, gray arrow 1) disappeared when 37-R was used as a competitor (Fig. 4B, gray arrow 1), suggesting that the 3′-end of

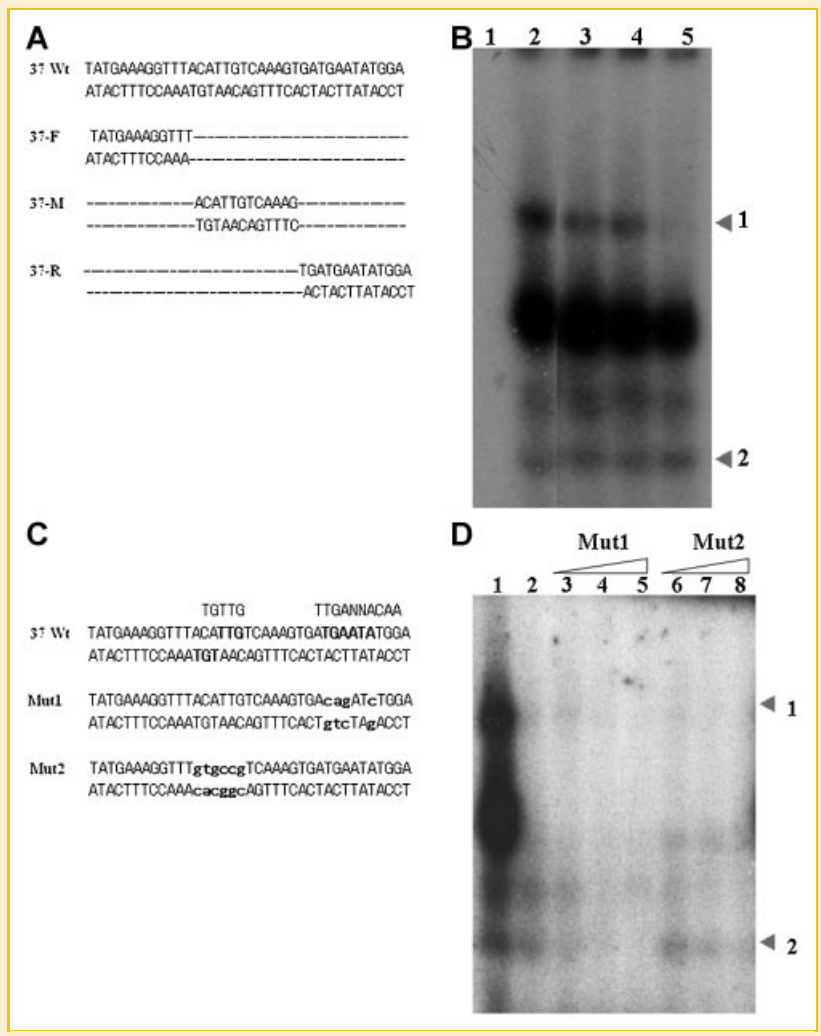

Fig. 4. Identification of the PARP-1 binding site at 37 mbr. A: Three truncated sequences which contain different part of 37 mbr were synthesized. 37-F contains 12 oligonucleotides located in the upstream of 37 mbr, while 37-R contains 13 oligonucleotides located in the downstream of 37 mbr. The intervening sequence of 37 mbr was named as 37-M. B: Competition assays with 37 mbr as the target. Lane 1 is free probe control without protein lysate; lane 2 shows the 37 mbr–protein complexes from Jurkat nuclear extracts; lane 3–5 are with 5 pmol unlabeled 37-F, 37-M, or 37-R as cold probes (competitive sequences), respectively. Gray arrows show protein–DNA complexes 1 and 2. C: Sequences of mutated probes. Comparison of PARP-1-binding sequences with 37 mbr indicates that there are two possible PARP-1-binding sites, mutations were made corresponding to these two possible sites (downstream-binding site as Mut1 and the intervening binding site as Mut2). D: Competition assays with mutated 37 mbr. Mut1 and Mut 2 were used as cold probes and incubated with Jurkat nuclear extracts. Gray arrows 1 and 2 indicate the previously identified protein–DNA complexes (Fig. 3). Lane 1 shows the binding of nuclear extracts of Jurkat cells to labeled 37 mbr without competitor; lane 2 shows competition with unlabeled 37 mbr; lanes 3–5 show competition with different concentration of unlabeled Mut1; and lanes 6–8 show competition with different concentration of unlabeled Mut2.

37 mbr may be important for PARP-1 binding. However, there is no obvious changes in complex 2 (Fig. 4B, gray arrow 2) when three truncated sequences were used as competitors. We speculate that the core-binding site for complex 2 may be separated into two fragments. To further determine the exact binding site of PARP-1 in 37 mbr, we mutated two potentially PARP-1-binding sites in 37 mbr, based on comparison of the 37 mbr sequence to several known DNA-binding sites of PARP-1 (Fig. 4C) [Zhang et al., 2002; Huang et al., 2004; Ambrose et al., 2007]. Both complexes 1 and 2 disappeared when Mut1 and Mut2 were used as competitors, respectively (Fig. 4D, gray arrow 1 and 2).

PARP-1 BINDS TO 37 MBR IN VIVO

To further determine whether PARP-1 binds to 37 mbr in vivo, we conducted a ChIP assay. Fig. 3 shows the PCR amplification product after the immunoprecipitation of the cross-linked chromatin with anti-PARP-1 antibody (Fig. 5, lane 3). In contrast, almost no PCR-amplified product was found following the immunoprecipitation of the cross-linked chromatin with purified rabbit IgG (Fig. 5, lane 4). These data support the binding of PARP-1 to 37 mbr in vivo.

OVEREXPRESSION OF PARP-1 REDUCES THE REGULATORY FUNCTION OF THE 37 MBR

To test the effects of PARP-1 on the regulatory function of the 37 mbr, we cotransfected PARP-1 expression plasmid pPARP31 with 37 mbr-luciferase reporter plasmid (Fig. 6A) into Jurkat, MCF-7, Nalm-6, and HEK 293 cells. The pL15TK plasmid was transfected together with the reporter plasmid as a control. The luciferase activity decreased to 54% (Jurkat), 56% (MCF-7), and 43% (Nalm-6; Fig. 6B) in the presence of PARP-1 overexpression. However, we did not observe this effect of PARP-1 on 37 mbr regulatory function in HEK 293 cells (Fig. 6B). These data indicate that PARP-1 has a negative effect on the 37 mbr regulatory function, and this effect may be cell type dependent.

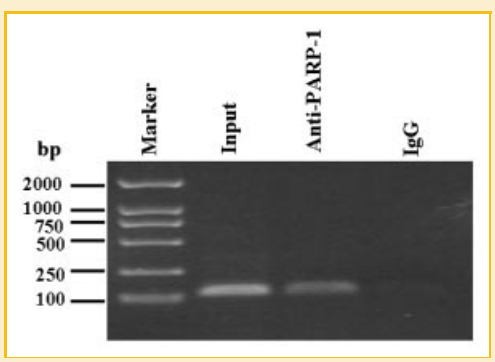

Fig. 5. Demonstration of PARP-1 as a 37 mbr-binding protein by ChIP. Cross-linked chromatin of Jurkat cells was isolated and immunoprecipitated with anti-PARP-1 antibody. DNA recovered without immunoprecipitation (input) was used as a positive control. The immunoprecipitation of the cross-linked chromatin with purified rabbit IgG was used as a negative control. PCR was performed using primers specific for the 37 mbr of BCL2 gene as described in the Materials and Methods Section. The amplified DNA fragments were visualized on a 1.5% agarose gel.

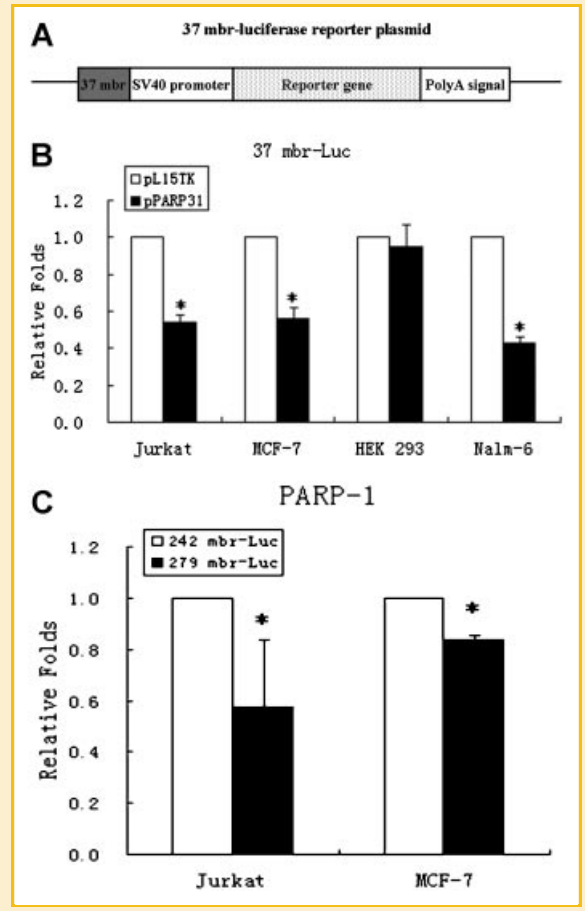

Fig. 6. Overexpression of PARP-1 reduces the 37 mbr regulatory function. A: 37 mbr-luciferase reporter construct is shown. B: Jurkat, MCF-7, HEK 293, and Nalm-6 cells were co-transfected with the PARP-1 expression plasmids (pPARP31) and the 37 mbr-luciferase reporter plasmids. pL15TK plasmids were used as a control. β-Galactosidase expression plasmids were co-transfected with the constructs described above to normalize the luciferase activity. C: Jurkat and MCF-7 cells were co-transfected the PARP-1 expression plasmids (pPARP31) with 279 mbr-luciferase or 242 mbr-luciferase reporter plasmids. β-Galactosidase expression plasmids were co-transfected with the constructs described above to normalize the luciferase activity. Error bars indicate the mean ± SD of three determinations, $^{*}P < 0.05$.

PARP-1 was not the sole component of the 37 mbr–protein complex, and it is assumed that its function may be affected by other trans-factors. To further confirm the relationship between PARP-1 and 37 mbr, we cotransfected pPARP31 expression plasmid with 279 mbr-luciferase reporter plasmid or 242 mbr-luciferase reporter plasmid into Jurkat and MCF-7 cells, respectively. The luciferase activity decreased to 58% (Jurkat) and 84% (MCF-7) in cells transfected with 279 mbr-luciferase reporter plasmid compared to that in cells transfected with 242 mbr-luciferase reporter plasmid (Fig. 6C).

OVEREXPRESSION OF PARP-1 DECREASES BCL2 GENE EXPRESSION

To determine the effects of PARP-1 on BCL2 expression, we ectopically expressed PARP-1 in Jurkat, MCF-7, and HEK 293 cells

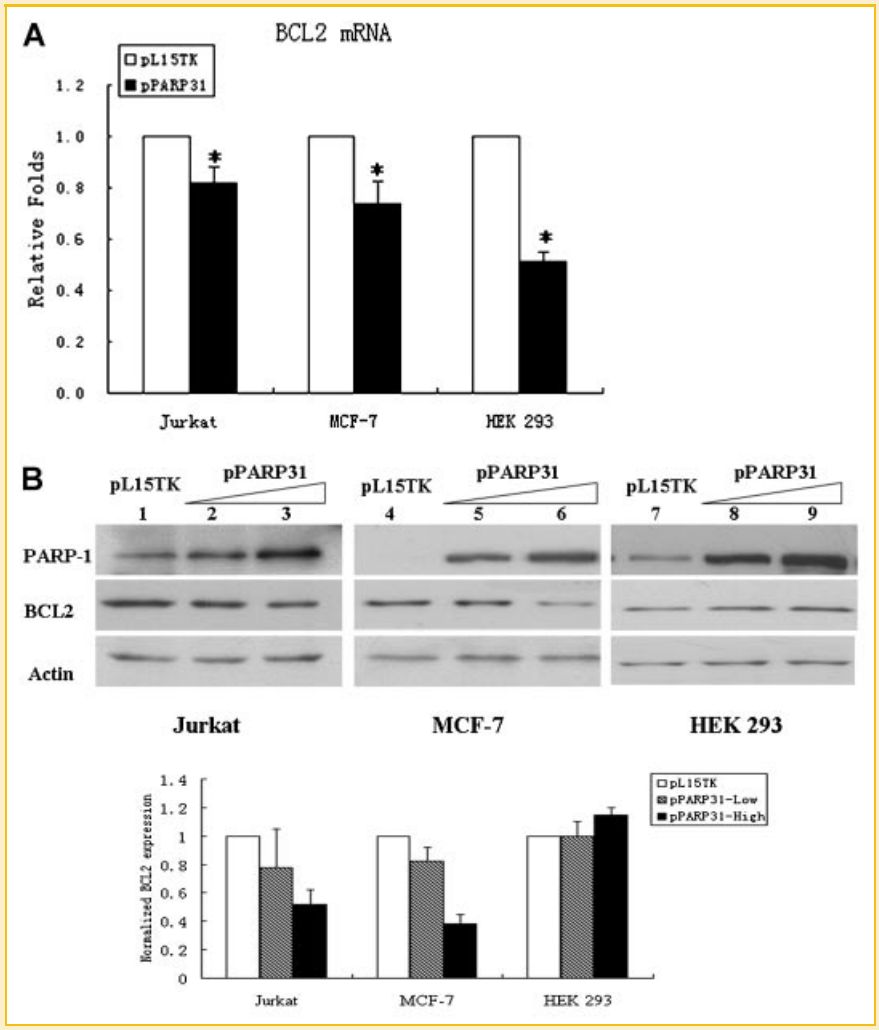

Fig. 7. Overexpression of PARP-1 decreases the BCL2 gene expression. Jurkat, MCF-7, and HEK 293 cells were transiently transfected with PARP-1 expression plasmid pPARP31 or control plasmid pL15TK, respectively. Cells were harvested 36 or 48 h after transfection. A: Real-time quantitative PCR analysis was performed to detect changes of BCL2 mRNA in different cell lines 36 h after transfection. Error bars indicate the mean ± SD of three determinations, $^{*}P < 0.05$. B: Western blot analysis was performed using an anti-PARP-1 polyclonal antibody or anti-BCL2 monoclonal antibody to detect the levels of PARP-1 and BCL2 proteins 48 h after transfection. β-Actin levels served as internal control. Normalized BCL2 expression is quantified by using Quantity One.

and monitored BCL2 expression at both mRNA and protein level. As shown in Fig. 7A, overexpression of PARP-1 resulted in the decrease of BCL2 mRNA expression in Jurkat, MCF-7, and HEK 293 cells. And BCL2 protein expression was decreased with increased expression of PARP-1 in Jurkat and MCF-7 cells. However, there were no obvious changes in BCL2 protein expression with increased expression of PARP-1 in HEK 293 cells (Fig. 7B).

KNOCKDOWN OF PARP-1 BY RNAI INCREASES BCL2 GENE EXPRESSION

To further verify the effect of PARP-1 on BCL2 expression, we performed miRNA-mediated knockdown of PARP-1 in MCF-7 and HEK 293 cells. As shown in Fig. 8A, BCL2 mRNA was increased concurrently with PARP-1 knockdown in MCF-7 and HEK 293 cells, and BCL2 protein expression was increased with decreased expression of PARP-1 in MCF-7 cells. However, there were no obvious changes in BCL2 protein expression with decreased expression of PARP-1 in HEK 293 cells. Due to cell death, we were unable to knockdown PARP-1 in Jurkat cells. Together, these results indicate that PARP-1 plays a role in regulation of BCL2 expression.

DISCUSSION

BCL2 expression is regulated at transcriptional, post-transcriptional, and post-translational levels and influenced by a variety of environmental and endogenous stimuli [Heckman et al., 2000, 2002; Schiavone et al., 2000; Wu et al., 2001; Donnini et al., 2004; Ma et al., 2007]. Our previous investigations demonstrated that the BCL2 mbr in the 3'-UTR is a *cis*-regulatory element [Zhang et al., 2006], and this regulatory element positively regulates BCL2 gene

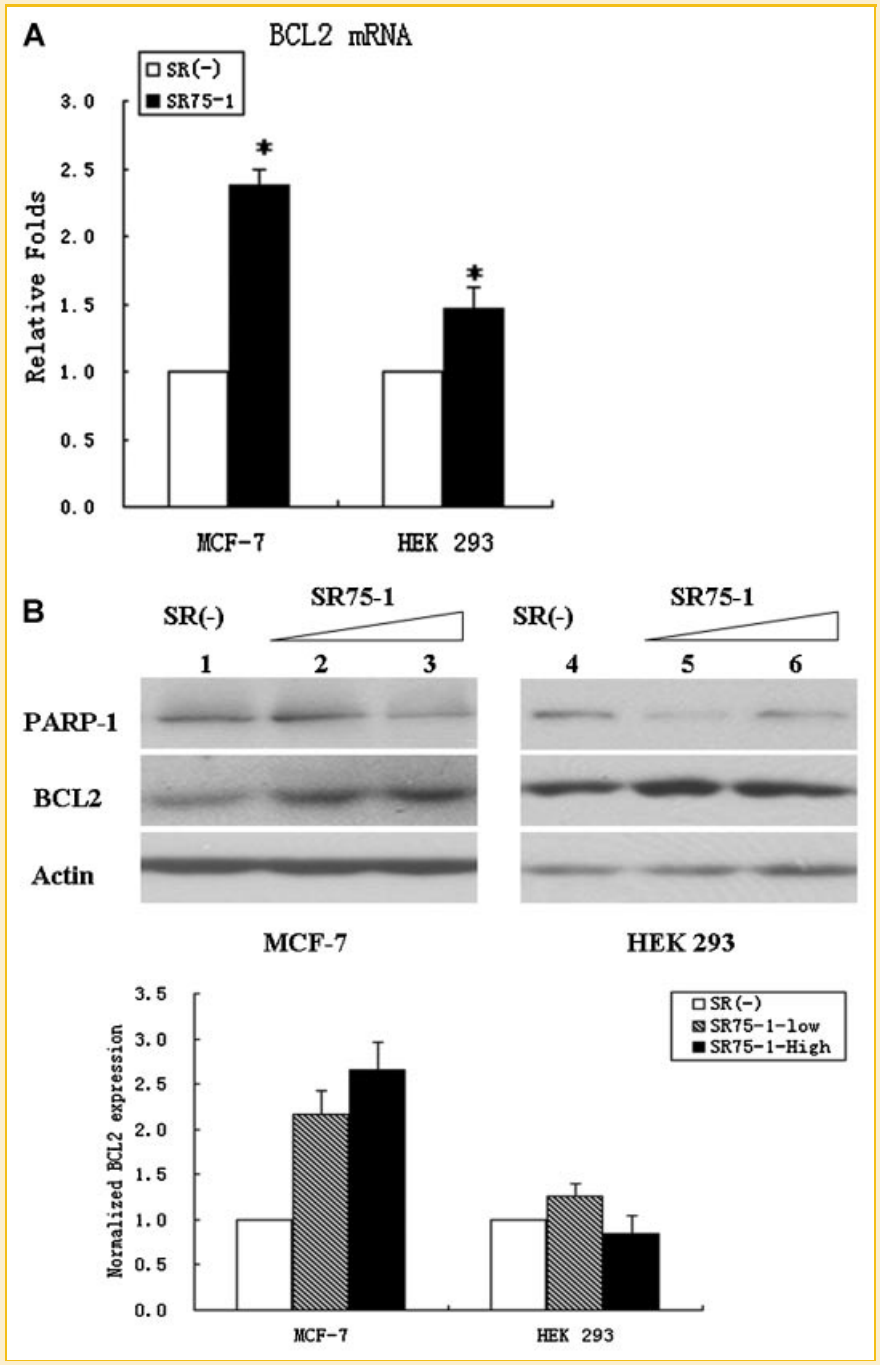

Fig. 8. Knock down of PARP-1 by RNAi increases the expression of BCL2. MCF-7 and HEK 293 cells were transiently transfected with PARP-1 RNAi plasmids SR75-1 or control plasmids SR(−). (A) Real-time quantitative PCR and (B) Western blot analysis were performed to measure BCL2 mRNA and protein, respectively. β-Actin levels served as internal control. Error bars indicate the mean ± SD of three determinations, $^{*}P < 0.05$. Normalized BCL2 expression is quantified by using Quantity One.

expression. In addition, we found that the regulatory function of mbr was closely associated with SATB1 [Ma et al., 2007]. SATB1, a nuclear matrix-attached protein, directly binds to 37 mbr located in the 3'-end of the mbr [Ramakrishnan et al., 2000]. It has also been reported that some uncharacterized additional factors in Jurkat nuclear extracts increased SATB1 affinity for the 37 mbr target 4- to 5-fold in vitro, and SATB1 is not the sole DNA-binding protein in this region [Ramakrishnan et al., 2000]. Therefore, the precise molecular mechanisms by which mbr regulates BCL2 gene expression are not fully understood. In this study, we found that (1) PARP-1 is a component of the protein complex which binds to 37 mbr in the BCL2 gene; (2) PARP-1 decreases the regulatory function of 37 mbr; (3) PARP-1 has a negative effect on the mRNA and protein expression of BCL2.

PARP-1 was reported to bind to the promoter region of the inducible nitric oxide synthase gene or centromeric α-satellite DNA, both of which contain a highly AT-rich sequence [Earle et al., 2000; Yu et al., 2006]. Here, we purified PARP-1 from DNA–protein complexes formed by 37 mbr containing high-AT levels and demonstrated that PARP-1 participates in the formation of DNA–protein complexes in vitro and in vivo. It is still unclear whether PARP-1 binds at this region directly or indirectly. Matrix attachment regions (SARs/MARs) usually exist in coding or flanking regions of genes and are in close proximity of the *cis*-acting elements required for gene transcription regulation [Cockerill et al., 1987]. The presence of MARs can result in long-range chromatin accessibility to transcription factors [Jenuwein et al., 1997]. In addition, base unpairing regions (BURs) are usually found in scaffolds or MARs, which contribute to the formation of the loop domain structure of chromatin and PARP was found to bind to BURs [Yanagisawa et al., 1996; Galande and Kohwi-Shigematsu, 1999]. Therefore, it is possible that PARP-1 binds directly to the 37 mbr, which contains an AT-rich sequence and is a MAR, via its cysteine residues within the zinc-finger domains as reported [Yu et al., 2006]. However, SATB1 has been identified as a 37 mbr direct binding protein. It functions as a landing platform to recruit proteins and regulates the genes by folding chromatin into loop domains [Cai et al., 2003]. Therefore, the possibility cannot be excluded that PARP-1 binds to 37 mbr indirectly by interacting with other proteins. For example, PARP-1 may be recruited by SATB1 to participate in the 37 mbr–protein complex's formation.

Using 37 mbr as a probe, Ramakrishnan et al. [2000] purified PARP-1 from the same DNA–protein complex as SATB1. Our results of gel supershift showed that PARP-1 participated in the formation of two 37 mbr–protein complexes, one of which contains both PARP-1 and SATB1 that has been proved to be involved in BCL2 regulation in our previous study. We speculate that the role of PARP-1 in regulation of BCL2 transcription may be related to SATB1.

We performed EMSA with unlabeled mutated oligonucleotides Mut1 and Mut 2 (Fig. 4C) as competitive probes to localize the PARP-1-binding site in the 37 mbr. Result showed that both DNA–protein complex 1 and DNA–protein complex 2 disappeared when Mut1 and Mut2 were used as competitive probes (Fig. 4D). This result indicates that either (1) PARP-1 may recognize the specific structures or base composition (such as the AT-rich region) rather than specific sequences as reported previously; or (2) PARP-1 may bind to 37 mbr indirectly and these two mutations do not affect the direct binding of other proteins.

PARP-1 has been reported to either repress or induce transcription of target genes by several different mechanisms [D'Amours et al., 1999; Kraus and Lis, 2003]. Our results of luciferase assay showed that PARP-1 has a negative effect on 37 mbr regulatory function in Jurkat, Nalm-6, and MCF-7 cells, but it has no effect on 37 mbr regulatory function in HEK 293 cells (Fig. 6B). Since the inner environments of different cell lines are different (e.g., the expression of SATB1 in Jurkat cells is relatively high, while its expression in MCF-7 cells is undetectable; Zhang et al., 2006), the effects of PARP-1 on 37 mbr regulatory may depend on the various inner environment of different cell lines. Moreover, real-time PCR showed that PARP-1 reduces BCL2 mRNA expression and Western blot assay showed that it has no obvious effects on BCL2 protein expression in HEK 293 cells. Since gene regulation occurs at different levels, such as chromatin level, transcriptional level, post-transcriptional level (mRNA stability), translational level, and post-translational level (protein stability), it is possible that PARP-1 affects BCL2 expression at post-transcriptional level (mRNA stability) and translational level or post-translational level (protein stability) in HEK 293 cells. This needs to be proved further.

In summary, our present study demonstrated that PARP-1 is a component of the 37 mbr–protein complexes and has a negative effect on the transcriptional activity of BCL2 gene. Together with our previous study, these findings provide an insight into regulatory mechanisms of BCL2 expression.

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