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RBP95, a Novel Leucine Zipper Protein, Binds to the Retinoblastoma Protein[1]

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We recently identified a novel cDNA encoding a retinoblastoma protein (pRb)-associated protein. It was named RBP95, which was composed of 838 amino acid residues with a calculated molecular size of 94,789 Da. Northern blot analysis showed a single mRNA of about 4.5 kb ubiquitously expressed in human tissues. RH mapping results showed that *RBP95* is mapped to chromosome region 16p11.2-11.1. Sequence analysis indicated that RBP95 contains a conserved pRb-binding motif LXCXE. Interaction between pRb and RBP95 was confirmed *in vivo* and *in vitro*. This interaction requires the LXCXE motif of RBP95 and the entire pocket region of pRb. Each point-mutant of the conserved amino acid residues in pRb-binding motif of RBP95 would destroy its interaction with pRb. RBP95 also contains a basic region leucine zipper and could homodimerize through its leucine zipper region. RBP95 was located in the nucleus with a special pattern when expressed as a GFP fusion in HeLa cells. All these findings suggested that RBP95, a new member of pRb-associated protein, may function as a regulation factor in the process of RNA polymerase II-mediated transcription and/or transcriptional processing.

Key Words: **pRb-associated protein; yeast two-hybrid system; RBP95; basic region leucine zipper; nuclear localization.**

The retinoblastoma (*Rb*) gene is well known as a tumor suppressor gene, and its product, 105-kDa retinoblastoma protein (pRb), is a negative growth regulator (1). pRb controls cellular proliferation and participates in critical determination among the cellular fates of cell cycle progression, temporary cell cycle arrest, quiescence, differentiation, senescence or apoptosis (2). Its functional inactivation accounts for the oncogenesis in over 60% of human tumors studied (3).

pRb is a nuclear phosphoprotein of which two known biochemical properties have been described: one is its intrinsic DNA-binding activity mapped to its C-terminal 300 amino acid residues; another is its ability to interact with several cellular proteins and a number of oncoproteins encoded by DNA tumor viruses (4). This interaction was mapped to the "pocket" domain: pocket A (amino acid 394 to 572), pocket B (amino acid 646 to 792) and the spacer between them (5). This "pocket" domain is very important, but is not sufficient to inhibit cellular proliferation. The C-terminal region in collaboration with the pocket region is also essential for the growth-suppressive activity of pRb (6).

pRb is a multifunctional component of a complicated signal transduction network called "RB pathway" (3, 7). Interactions of pRb with various cellular proteins play very important roles in its function. Inactivation of any component of pRb pathway is sufficient to perturb normal growth control. Thus, more attention has been focused on identifying the proteins functionally interacting with pRb. More than 40 proteins have been shown to interact with pRb either *in vivo* or *in vitro* (8). Most of these interactions require the pocket domain of pRb, and some of these proteins contain a conserved LXCXE pRb-binding motif (9).

We used the whole C-terminus of human retinoblastoma protein (amino acid residues 377–928) as bait to screen a human fetal brain MATCHMAKER cDNA library. In this paper, we described the identification and cloning of a novel cDNA sequence encoding a 95-kDa new member of pRb-associated protein (RBP95). RBP95 is a basic region leucine zipper protein, and it could form homodimer through its leucine zipper region. In addition, we confirmed the specific interaction between pRb and RBP95 *in vitro* and defined the definite binding site of RBP95 and pRb. We showed that

Abbreviations used: AD, activation domain; DBD, DNA binding domain; CMV, cytomagalovirus; DAPI, 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; IP, immunoprecipitation; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; Rb, retinoblastoma; RBP95, 95 kDa retinoblastoma protein-binding protein; RH, radiation hybrid; RT-PCR, reverse transcription-polymerase chain reaction.

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RBP95 is extensively expressed in different human tissues. Furthermore, we identified the chromosomal and subcellular localization of RBP95.

MATERIALS AND METHODS

Yeast two-hybrid screening and cDNA cloning of RBP95. Two-hybrid screening was carried out essentially as described in detail elsewhere (10), using the partial human retinoblastoma protein as bait. pGBT9-Rb vector was used to express a partial human retinoblastoma protein (377–928 aa) fused to the C-terminus of GAL4 DNA-binding domain (DBD). Human fetal brain MATCHMAKER cDNA library (Clontech) was constructed in the GAL4 activation domain (AD)-encoding vector pGAD10. The bait and the library were co-transformed into *Saccharomyces cerevisiae* strain HF7C (11). Transformants were selected on yeast drop-out minimal medium (−Leu, −Trp, and −His). *His*$^+$ colonies were picked and tested for the expression of the second reporter gene *LacZ* through colony lift β-galactosidase activity filter assay (12). cDNA plasmids were isolated from each positive yeast clone (*His*$^+$, *β-gal*$^+$) and the interaction was reconfirmed in yeast strain SFY526 (13). An insert cDNA fragment, designated BP125 (representing a portion of RBP95), was isolated from the two-hybrid screen. Full-length cDNA of *RBP95* was cloned from a HeLa cDNA library (in λgt10, Clontech) by standard DNA hybridization technique (14). Positive cDNA insert fragment was cloned and sequenced. The database search and alignments were performed with BLAST search of NCBI (15). Its amino acid sequence deduction and protein structure analysis were performed with the DNAStar, SMART (16), and some tools in the ExPASy World Wide Web molecular biology server.

In vitro translation and in vitro pRb-binding assay. C-terminal pRb (377–928 aa) coding sequence and HA-tagged RBP95 partial coding sequence (74–292 aa) were cloned into plasmid pGEM-7Z(+) (Promega) respectively. *In vitro* translation reaction was performed using a TNT Coupled Reticulocyte Lysate Systems (Promega) according to the User Manual. [^{35}S]-methionine was incorporated into the protein products.

8 μl of [^{35}S]-methionine labeled-pRb was mixed with 4 μl of [^{35}S]-HA-RBP95 in 200 μl IP Buffer (50 mM Tris–Cl (pH 7.5), 1 mM EDTA, 0.1% Triton X-100, 200 mM NaCl and 10% glycerol) containing protease inhibitors. The mixture was incubated with 2 μg of a monoclonal anti-HA antibody 12CA5 (Boehringer) and 20 μl of protein G agarose beads (Boehringer) for 2 h at 4°C with constant rocking. Pellets were washed three times with IP buffer, and the bound proteins were analyzed by SDS–PAGE followed by autoradiography.

Northern blot analysis cDNA fragment *BP125* was labeled with [α-^{32}P]-dCTP using Random Primers DNA Labeling System (GIBCO BRL). Human multiple tissue Northern blots and multiple cancer cell lines blot (Clontech) were hybridized in ExpressHyb hybridization solution (Clontech) according to the User Manual. As a control, filters were rehybridized with β-actin cDNA probe.

RT-PCR. Human fetal brain poly(A)$^+$ RNA was purchased from Clontech. Total RNA of HeLa cells was extracted by the TRIzol reagent (GIBCO BRL) and the poly(A)$^+$ RNA was purified by polyATtract mRNA isolation system (Promega). Two primers were designed for RT-PCR derived from the obtained sequences (RBP95RT1: 5′-GCCATGTCTGGGCCAGGCAAC-3′ and RBP95RT2: 5′-CCCCACCTCTCCAGCTCCAGTCTG-3′). First strand cDNA was synthesized with the oligo-$(dT)_{18}$ anchor primer and MMLV reverse transcriptase supplied in the Advantage RT-for-PCR kit (Clontech).

Mutagenesis and two-hybrid experiments. Human cDNA encoding the full-length RBP95 ORF was cloned into the *Eco*R I and *Bam*H I sites of pGAD424 and pGBT9 in frame to obtain pGAD424-RBP95 and pGBT9-RBP95. RBP95-ΔBLZ (deleted the amino acid 202–296), RBP95L109S (RBP95 with a Leu109 → Ser substitution), RBP95C111M (RBP95 with a Cys111 → Met substitution) and RBP95E113Q (RBP95 with a Glu113 → Gln substitution) were prepared by PCR-mediated site-directed mutagenesis (17), and cloned into pGAD424 or pGBT9 in frame. Truncated pRb (377–640), pRb (641–792), pRb (793–928) and pRb (379–792) were amplified in PCRs, and constructed in *Eco*R I and *Bam*H I sites of pGBT9. All these plasmids were subjected to DNA sequencing for the confirmation of correct construction. Various pairs of the pGAD424 and pGBT9 plasmids were cotransformed into yeast reporter strain HF7C to test for their abilities to grow on plates lacking histidine, or cotransformed into another yeast reporter strain SFY526 to test for the β-galactosidase activity. Liquid culture β-galactosidase assay with ONPG as substrate was performed as described in protocol: MATCHMAKER Two-Hybrid System (PT1265-1) (Clontech).

Chromosomal localization of human RBP95 by RH mapping. For RH mapping (Radiation hybrid mapping), we used the Stanford G3RH panel (Stanford Research Genetics Inc., Huntsville AL) which contains 83 human/hamster hybrid clones. PCR primers: RH1 (5′-CCCTG CCTAC TGGCT CACAA AT-3′) and RH2 (5′-GGAAG GGGCC TGATT AGAAT AG-3′) were designed according to the sequence in the 3′-UTR of *RBP95*. 25 ng DNA for each of the 83 hybrid clones were used for PCR. After denaturation at 94°C for 4 min, thirty-five cycles of amplification were performed: 94°C for 25 s, 57°C for 30 s and 72°C for 30 s. PCR results were analyzed with the tools in the Radiation Hybrid Mapper Server at Stanford Human Genome Center (http://www-shgc.stanford.edu/RH/rhserverformnew.html) and Genome Database (http://www.gdb.org/jmap/queryByPosn.html) to obtain the result of RBP95 chromosomal localization. This PCR was repeated three times.

Subcellular localization of GFP-RBP95 in human cells. The *RBP95* cDNA-containing entire ORF was cloned into the pEGFP-N_2 vector (18, 19) (Clontech) designed to express a fusion between the C-terminal of RBP95 and the N-terminal of enhanced green fluorescent protein (EGFP) under the control of CMV promoter. Plasmids expressing GFP alone (pEGFP-N_2) or the RBP95-GFP fusion (pEGFP-N_2-RBP95) was transfected into the HeLa cell using a calcium-phosphate transfection method (20). Approximately 24 h after the transfection, transfected cells were washed with PBS, and fixed in 4% paraformaldehyde. The nuclei were stained by DAPI. Cells were imaged by fluorescent microscopy.

Nucleotide accession number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence database with Accession No. AF122819.

RESULTS

Isolation of RBP95 as a New Member of pRb-Associated Protein by Yeast Two-Hybrid Screen

We set out to identify more pRb-associated cellular proteins. As a starting point, we employed the yeast two-hybrid system. Since the C-terminal region and the intact A/B pocket domain of pRb are the most important regions in function, we used the whole C-terminus of human retinoblastoma protein (amino acid residues 377 to 928) as bait. Rb bait plasmid pGBT9-Rb and pGAD10 plasmids containing the human fetal brain cDNA library were cotransformed into yeast strain HF7C. Interactions between pRb and pRb-associated proteins were detected by the growth on minimal medium lacking histidine and by production of β-galactosidase activity (Fig. 1). Library plasmid in one of those positive clones (*His*$^+$, *β-gal*$^+$), *pGAD10-BP125*, contained a 657-bp insert sequence encoding a

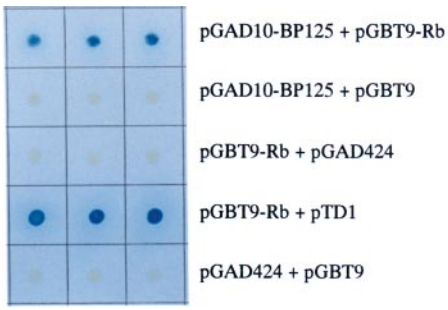


FIG. 1. Specific interaction between pRb and BP125 in yeast two-hybrid system. The filter shows the β-galactosidase activity obtained from cotransformed SFY526 cells picked on plates lacking Trp and Leu. BP125 was identified in the yeast two-hybrid library screen and was expressed as a fusion protein with the GAL4 activation domain in pGAD10. Amino acids 377–928 of pRb were fused to the GAL4 DNA binding domain in pGBT9 (pGBT9-Rb). pTD1, containing the fusion of SV40 large T-antigen and GAL4 activation domain and used as a positive control. β-Galactosidase activity of SFY526 transformants was assayed as described (12). Each experiment was repeated three times as shown.

predicted 219 amino acids peptide. Northern blot analysis indicated that full-length cDNA of *BP125* was about 4.5 kb. We screened a HeLa cDNA library (in λgt10) to clone the entire coding sequence of *BP125*. A positive clone containing ~4.5-kb cDNA insert was obtained, and the whole sequence was determined.

Characterization of RBP95 Sequence

The 4305-bp cDNA sequence contains a 2517-bp ORF (Fig. 2A and GenBank Accession No. AF122819). The putative initiation ATG occurs at nucleotide 97, which is preceded by an in-frame stop codon TAG located 90 bp upstream. The in-frame downstream stop codon occurs at nucleotide 2611, and the potential poly(A)$^+$ adenylation signal (AATAAA) is found in the 3′-UTR at nucleotide 4228. The predicted translation product of this novel gene is composed of 838 amino acids, with a calculated molecular size of 94,789 Da, so we named this protein RBP95 (95 kDa pRb-binding protein) (Fig. 2B).

Sequence analysis revealed some distinctive features of this putative protein (Fig. 2). First, RBP95 contains a conserved pRb-binding motif Leu-X-Cys-X-Glu (LXCXE) (109~113 amino acids) followed by a casein kinase II phosphorylation site, which was conserved in many other pRb-associated proteins, such as RBP-1, SV40 large T-antigen and HPV16 E7 protein (21) (Fig.

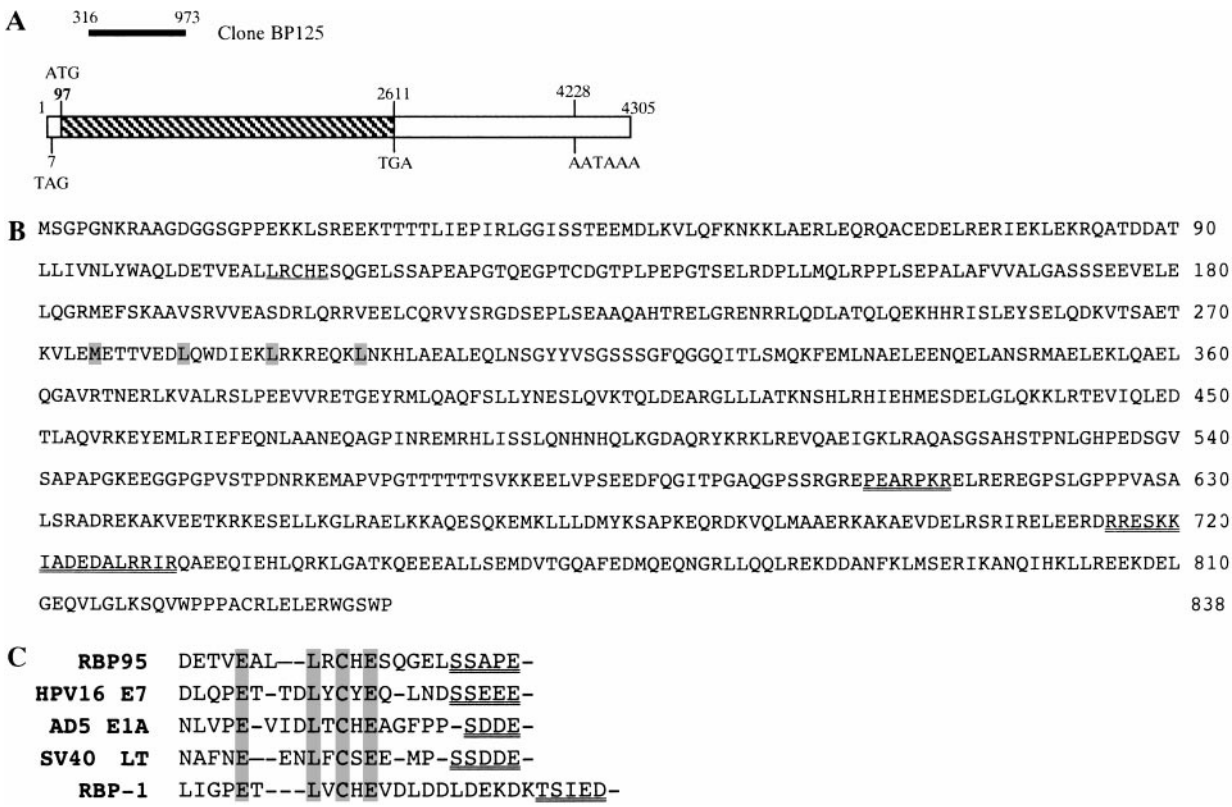


FIG. 2. *RBP95* cDNA and protein. (A) Schematic representation of the *RBP95* cDNA. The ORF and region encompassed by the clone *BP125* insert are indicated. This sequence is available in GenBank with Accession No. AF122819. (B) Deduced amino acid sequence of RBP95. The conserved pRb-binding motif is underlined. Nuclear localization signals are indicated by double lines, and the conserved leucine in basic leucine zipper are shaded. (C) Alignment of the pRb-binding region in RBP95 with other pRb-associated proteins. The conserved pRb-binding motif LXCXE is shaded, and casein kinase II phosphorylation sites are doubly underlined.

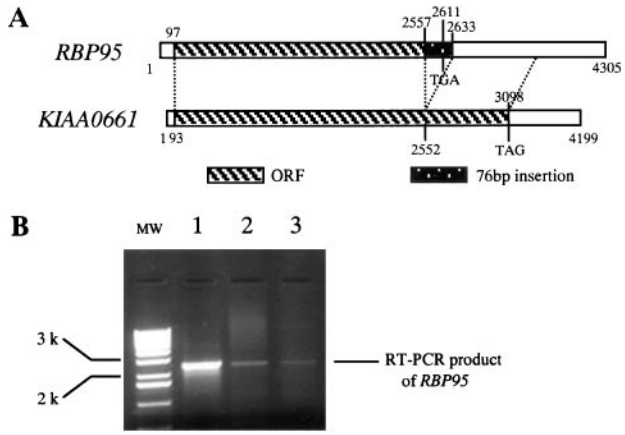


FIG. 3. RT-PCRs for the confirmation of the RBP95 transcripts. (A) Schematic comparison between cDNA of RBP95 and KIAA0661. (B) RT-PCR results. RT-PCRs were performed as described under Materials and Methods. Lane 1, using *pBSK-RBP95* plasmid as a positive control; lane 2, using poly(A)$^+$ RNA from the fetal brain as template; lane 3, using poly(A)$^+$ RNA from the HeLa cell lines as template.

2C). Second, using the PSORT program, two basic regions (606–612 amino acids and 715–731 amino acids) containing potential bipartite nuclear localization signals (22) were found in RBP95, indicating that RBP95 might be a nuclear protein. Third, analyzed with SMART program, a basic region leucine zipper (201–296 aa) (23), an I/LWEQ domain (149–304 aa) (24) and a spectrin repeat (252–354 aa) (25) were also found in this predicted RBP95 protein.

Interestingly, during the process of cloning the full-length cDNA of RBP95, a 4.2 kb human cDNA sequence encoding KIAA0661 protein which matched our cDNA sequence almost exactly was submitted by Ishikawa to GenBank (Accession No. AB014561) (26). Comparing these two cDNA sequences, we found that a 76-bp cDNA fragment of *RBP95* (2557–2633 nt) did not exist in AB014561. As for AB014561, the insertion site of this short sequence was between nt 2552 and nt 2553. The incorporation of this 76-bp insertion in the transcript results in translation termination due to the presence of an in-frame stop codon, therefore RBP95 and KIAA0661 proteins have different C-termini (Fig. 3A). To confirm the existence of *RBP95* transcript, which contains the 76 bp insertion, we designed a RT-PCR primer (RBP95RT2) within this insertion region. Together with another primer located in the 5′-translation initiation region (RBP95RT1), RT-PCRs were performed. As shown in Fig. 3B, the ~2.5-kb transcript of *RBP95* was detected in the RT-PCR products from human fetal brain and HeLa cell poly(A)$^+$ RNA. Consensus splice donor site and acceptor site were found at the 3′- and 5′-ends of the insertion respectively, so we inferred that this 76-bp insertion was an unspliced intron. It suggested that there might be different splicing patterns, and our *RBP95* and Ishikawa's KIAA0661 cDNA representing the corresponding products.

When compared with other proteins using BLAST search (15), RBP95 shared the highest sequence similarity with myosin heavy chains. RBP95 sequence exhibited 19% identity over a 663 amino acid region (175–809 aa) with *Homo sapiens* myosin heavy chain. RBP95 also showed homology to troponin, tropomyosin and intermediate filament to some degree. All these proteins contain similar secondary structures: they have been shown to form long coiled-coils. DNAStar secondary structure analysis also indicated that the predicted RBP95 protein had the tendency to be a coiled-coil protein.

RBP95 Is Ubiquitously Expressed in Human Tissues

Expression of *RBP95* mRNA was detected by Northern blots. As shown in Fig. 4, a single band of ~4.5 kb was detected in all selected normal human tissues and cancer cell lines. But this band was not detected in 20 μg of total RNA obtained from the same human tissues and some cultured human cell lines (data not shown).

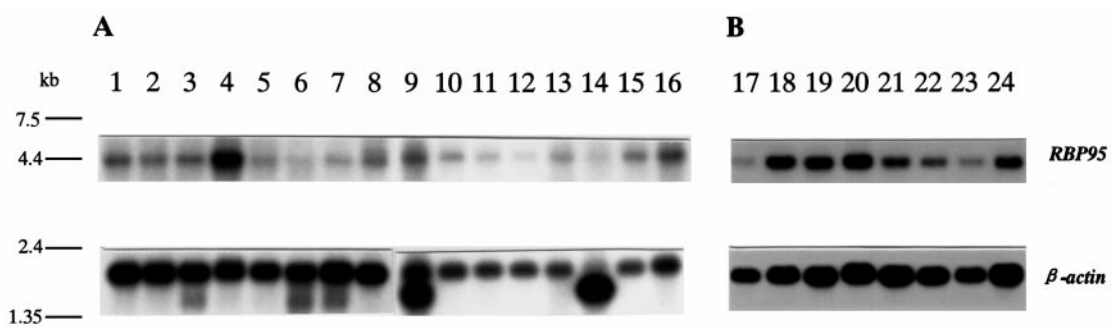


FIG. 4. Northern analysis of poly(A)$^+$ RNA from various human tissues (A) and human cancer cell lines (B). The blots were hybridized with RBP95 cDNA (top) or β-actin cDNA (bottom) as described under Materials and Methods. 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocyte; 9, heart; 10, brain; 11, placenta; 12, lung; 13, liver; 14, skeletal muscle; 15, kidney; 16, pancreas; 17, promyelocytic leukemia HL-60; 18, HeLa cell S3; 19, chronic myelogenous leukemia K-562; 20, lymphoblastic leukemia MOLT-4; 21, Burkitt's lymphoma Raji; 22, colorectal adenocarcinoma SW480; 23, lung carcinoma A549; 24, melanoma G361.

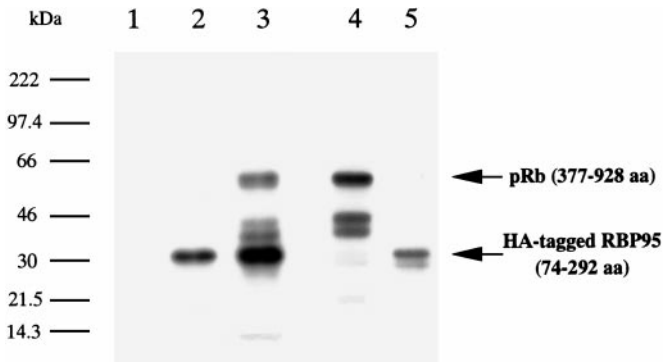


FIG. 5. RBP95 and the C-terminus of pRb interact directly *in vitro.* Binding of HA-tagged RBP95 with pRb was examined by coimmunoprecipitation assay described under Materials and Methods. pRb showed no nonspecific binding to the anti-HA antibody 12CA5 (lane 1), *in vitro* translated HA-tagged RBP95 fragment was immunoprecipitated by 12CA5 antibody (lane 2), and pRb could be pulled down together with HA-RBP95 when immunoprecipitated by 12CA5 antibody (lane 3). 1/10 input of *in vitro* translated proteins are indicated in lane 4 (pRb) and lane 5 (HA-RBP95). The arrows labeled pRb and HA-RBP95 at the right margin represent the migration position of the full-length translation products of pRb (377-928aa) and HA-RBP95 (74-292 aa).

These results suggested that *RBP95* is expressed extensively but at a relatively low level. The quantity of *RBP95* transcripts was varied: it was most abundant in testis, heart, and pancreas; but was only detectable in lung, skeletal muscle and small intestine (Fig. 4A). Consistent with it, the expression level of *RBP95* was very low in lung cancer cell line A549 (Fig. 4B).

Chromosomal Localization of RBP95 Gene

As described under Materials and Methods, PCRs were carried out. A 310-bp unique band could be generated in human genomic DNA-positive control reaction. PCR results were presented with 0 or 1 in order, 0 representing no positive PCR product and 1 representing a positive amplification. These raw scores were sent to the Radiation Hybrid Mapper Server at the Stanford Human Genome Center, which returned a framework marker linked to *RBP95*. After analyzed in GDB database, we determined that RBP95 was located to the chromosome region 16p11.2-11.1.

RBP95 Could Interact with pRb in Vitro

To determine whether there is a direct interaction between RBP95 and pRb, pRb (377–928 aa) and HA-tagged RBP95 (74–292 aa) were synthesized in the coupled transcription and translation system with [^{35}S]-methionine labels. Then, coimmunoprecipitation assays were performed. Upon incubation with [^{35}S]-HA-RBP95, [^{35}S]-pRb peptide was coimmunoprecipitated by HA monoclonal antibody (Fig. 5). HA-tagged RBP95 could also be pulled down with pRb by monoclonal anti-Rb antibody IF8 (data not shown). These results confirmed that RBP95 could interact with pRb directly, and also indicated that it was the N-terminus of RBP95 containing the potential pRb-binding motif leading to its interaction with retinoblastoma protein.

RBP95 Binds to the Pocket Domain of pRb through Its Conserved LXCXE Motif

Normally, pRb-binding motif is very important for LXCXE-containing pRb-associated proteins to interact with pRb. The conserved LXCXE motif could also be

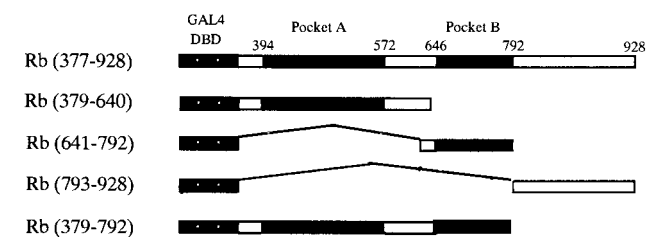
TABLE 1

RBP95 Binds to the Pocket Domain of pRb through Its Conserved LXCXE Motif

A

DNA binding domain fusion	Activation domain fusion	β-Galactosidase activity: Filter assay (blue/white)	β-Galactosidase activity: Units of β-galactosidase in liquid assay
Rb (377–928)	SV40 large T-antigen	Blue	135.2 ± 4.56
	BP125	Blue	30.8 ± 1.02
	RBP95	Blue	14.9 ± 0.2
	RBP95L109S	White	0.18 ± 0.09
	RBP95C111M	White	0.09 ± 0.016
	RBP95E113Q	White	0.15 ± 0.009
GAL4 DBD	GAL4 AD	White	0

B



DNA binding domain fusion	Activation domain fusion	β-Galactosidase activity: Filter assay (blue/white)	β-Galactosidase activity: Units of β-galactosidase in liquid assay
Rb (377–928)	RBP95	Blue	14.9 ± 0.2
Rb (379–640)		White	0
Rb (641–792)		White	0.09 ± 0.012
Rb (793–928)		White	0.03 ± 0.014
Rb (379–792)		Blue	23.76 ± 3.84
GAL4 DB	GAL4 AD	White	0

Note. The yeast reporter strain SFY526 was cotransformed with pairs of recombinant plasmids as shown in the tables. The level of β-galactosidase activity was determined as described under Materials and Methods. Values are means of triplate determinations. (A) Interaction between full-length RBP95 or RBP95 mutants and pRb. (B) Interactions between full-length RBP95 and truncated pRb. Schematic diagram of truncated pRb was shown.

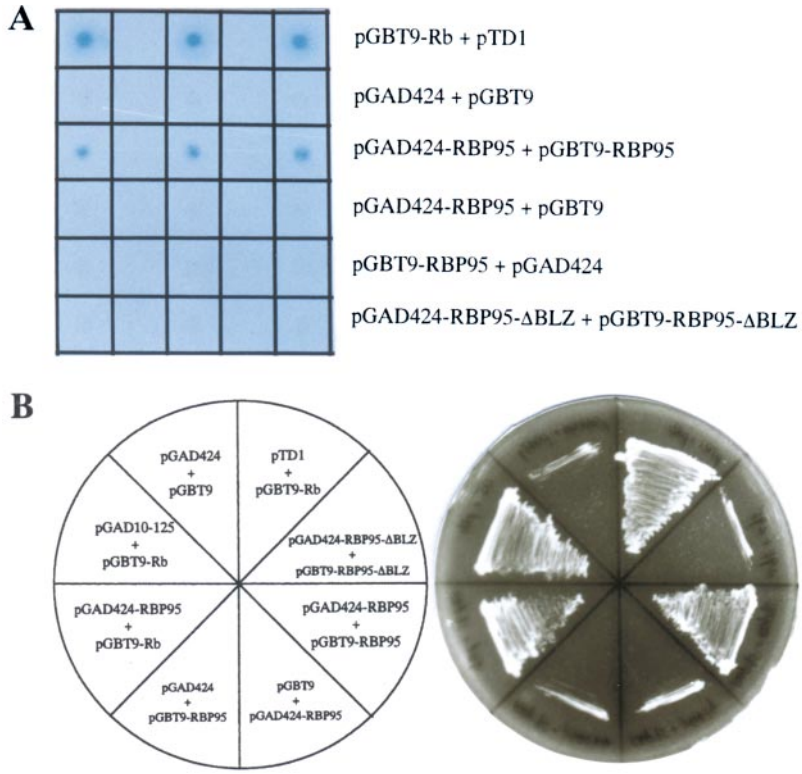


FIG. 6. Homodimerization of RBP95 in two-hybrid system. Yeast cells were cotransformed by different pairs of plasmids as indicated. (A) The filter shows the β-galactosidase activity obtained from cotransformed SFY526 cells picked on plates lacking Trp and Leu. Each experiment was repeated three times as shown. (B) Growth ability of HF7C transformants on minimal medium deficient in Trp, Leu and His. Self-interactions exist in RBP95 but not RBP95-ΔBLZ.

found in the N-terminal region of RBP95 (109–113 aa). So we designed a series of experiments to define whether RBP95 interact with pRb through the LXCXE motif. Full-length RBP95 and three RBP95 point-mutants: RBP95L109S, RBP95C111M and RBP95E113Q were constructed in pGAD424 and cotransformed with pRb bait plasmid into yeast strain HF7C, respectively. Only the cotransformant of full-length RBP95 with pRb could grow on the SD plates lacking histidine, and that of the three mutants could not (data not shown). We also assayed the liquid β-galactosidase activity of these transformants in SFY526 strain. As shown in Table 1A, full-length RBP95 could interact with pRb, although this interaction was weaker than BP125 clone; but almost no β–galactosidase activity was detected in RBP95L109S, RBP95C111M and RBP95E113Q transformants. These results showed that these RBP95 mutants lost the pRb-binding ability, so we can conclude that the conserved LXCXE motif in RBP95 was absolutely important for its binding to pRb.

As we known, there are several separate protein-binding sites located in the N-terminal, C-terminal and the middle part "pocket" domain of pRb (27). Many LXCXE-containing pRb-associated proteins interact with pRb through the entire pocket region (pocket A, pocket B and the spacer region) of pRb. Our experiments confirmed that the interaction between RBP95 and pRb also required the entire structure of this pocket region. In yeast two-hybrid system, full-length RBP95 could bind to the truncated form of pRb that retained the entire pocket region (379–792) and this interaction was even stronger than that of RBP95 with pRb (377–928), but RBP95 was not able to bind to truncated pRb only containing pocket A (379–640), pocket B (641–792) or C-terminus (793–928) respectively (Table 1B).

RBP95 Could Form Homodimer through Its Leucine Zipper Region

Many leucine zipper proteins can form dimer and act as transcription factors through their basic region. Sequence analysis shows that RBP95 has a basic region leucine zipper; could this protein form homodimer through its leucine zipper region? We constructed full-length RBP95 and mutant form RBP95-ΔBLZ (deleted the basic region leucine zipper) into pGAD424 and pGBT9 respectively. As shown in Fig. 6, pGAD424-RBP95 and pGBT9-RBP95 cotransformed SFY526 possessed the β–galactosidase activity, and their HF7C transfomants could grow on the SD plates lacking histidine; but no interactions were detected in pGAD424-

ΔBLZ and pGBT9-ΔBLZ cotransformants neither in SFY526 nor in HF7C. This result inferred that RBP95 could form homodimer and this homodimerization required the leucine zipper region.

RBP95 Was Located in the Nucleus in a Nonhomogeneous Pattern

The pRb-associated protein RBP95 has a basic region leucine zipper, and two potential bipartite nuclear localization signals exist in RBP95, so we are interested in whether RBP95 is really located in the nucleus and acts as a transcription factor. We constructed an expression plasmid pEGFP-N_2-RBP95, which could express an RBP95-GFP fusion protein. HeLa cells were transiently transfected with plasmids expressing either GFP alone or RBP95-GFP. Twenty-four hours after the transfection, cells were fixed and stained by DAPI. Subsequently, cells were imaged using fluorescence microscopy. Cells expressing the GFP protein alone displayed a diffuse and uniform cellular fluorescence. By contrast, cells expressing RBP95-GFP exhibited nuclear fluorescence (Fig. 7). Furthermore, the nuclear distribution pattern of RBP95 was very special: RBP95-GFP localized in the nucleus in a nonhomogeneous pattern; it was excluded from the nucleoli and appeared as tens of discrete speckles.

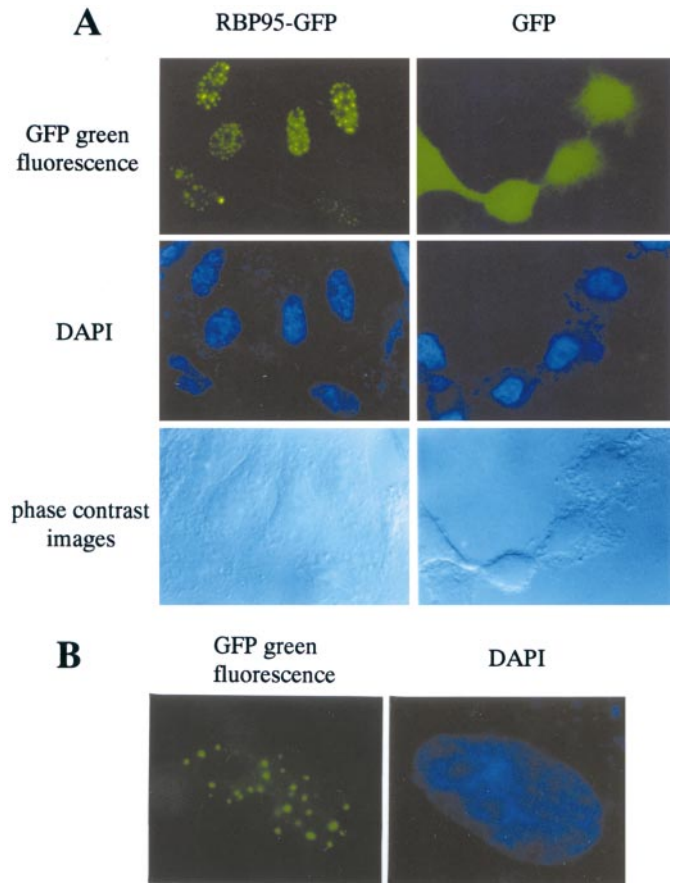


FIG. 7. RBP95 is a nuclear protein. HeLa cells were transfected with pEGFP-N2-RBP95 and pEGFP-N2, respectively, and imaged using fluorescent microscopy. (A) Comparison the subcellular localization of RBP95-GFP and GFP. The upper images are the GFP green fluorescence of transfected HeLa cells, the middle are DAPI blue fluorescence indicating the nuclear region, and the lower are phase-contrast images. (at ~30× magnification) (B) Fluorescent Image of one pEGFP-N2-RBP95 transfected cell at ~120× magnification.

DISCUSSION

In this work, we reported the identification and characterization of a cDNA, *RBP95,* encoding a novel member of pRb-associated proteins. We demonstrated the interaction of pRb with RBP95 *in vivo* and *in vitro.* In addition, we preliminarily analyzed the subcellular localization of RBP95. RBP95 has a special distribution pattern: It is concentrated in tens of speckles in the nucleus but does not exist in nucleoli. This kind of nuclear localization pattern had also been seen in several Ring finger proteins such as topors (28), ICPO (29), and some RNA 3′-processing factors (30). To the best of our knowledge, important components of the machinery for transcription, replication and splicing are all confined to certain nuclear matrix-associated subnuclear domains. With apparently organized nucleus architecture, localization studies have shown that pre-mRNA splicing factors are concentrated in 20–50 nuclear speckles in mammalian cell nucleoplasm, while DNA replication and transcription occur at several hundred or several thousand discrete nonoverlapping sites dispersed throughout the nucleoplasm (31). So, considering its distribution pattern in the nucleus, RBP95 is likely related to mRNA splicing factors. As a candidate target of pRb, how this interaction happens and how RBP95 functions? In performing its important physiological functions, pRb at least partly relies on its ability to interact with other cellular proteins that can affect transcription (27). Sequence analysis shows that RBP95 has a basic region leucine zipper, and our experiment indicated that RBP95 could form homodimer through this leucine zipper region, so it may possess the DNA-binding ability that is needed by most of the nuclear transcription factors. Basing on all these results, it may be hypothesized that RBP95 is involved in RNA polymerase II-mediated transcription and/or transcriptional processing.

Predicted RBP95 protein also shows sequence homology to some fibrous proteins such as myosin, intermediate filaments. Like these proteins, RBP95 is predicted to form long coiled-coils. Moreover, two domains which are capable of binding to F-actin and bundle actin filaments, I/LWEQ domain (24) and spectrin repeat (25), exist in the RBP95 protein. These findings imply that RBP95 may be related to the nuclear matrix. Taken together, RBP95 may probably associate with nuclear matrix at definite sites and contribute to

the assembling of the multicomponent machine of transcription and/or splicing. Studies designed to better define the localization of RBP95 in subnuclear domains should be done to determine whether it is involved in transcription or mRNA splicing or both. Another particular interest is whether RBP95 serves as a physiological target of pRb action and how it functions if it does. Our data may contribute to the understanding of the physiological roles of RBP95.

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