Dual Retinoblastoma-binding Proteins with Properties Related to a Negative Regulator of Ras in Yeast*

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The retinoblastoma protein (Rb) interacts with multiple cellular proteins that mediate its cellular function. We have identified nine polypeptides that bind to the T-binding domains of Rb using an Rb affinity resin. RbAp48 and RbAp46 are quantitatively the major Rb-associated proteins purified by this approach. RbAp48 was characterized previously and was found to be related to MSI1, a negative regulator of Ras in the yeast Saccharomyces cerevisiae. Here we report the cloning and characterization of RbAp46. RbAp46 shares 89.4% amino acid identity with RbAp48. The internal WD repeats, which are found in a growing number of eukaryotic proteins, are conserved between RbAp46 and RbAp48. Like RbAp48, RbAp46 forms a complex with Rb both in vitro and in vivo and suppresses the heat-shock sensitivity of the yeast RAS2Val-19 strains. We have also isolated the murine cDNA homologs of RbAp48 and RbAp46. Although both mRNA can be detected in all mouse tissues, their mRNA levels vary dramatically between different tissues. No significant differences were observed in the expression patterns of these genes in most tissues except thymus, testis, and ovary/uterus, in which 2-fold differences were observed. Interestingly, the mouse and human RbAp48 amino acid sequences are completely identical, and the mouse and human RbAp46 differ only by one conserved amino acid substitution. These results suggest that RbAp48 and RbAp46 may have shared as well as unique functions in the regulation of cell proliferation and differentiation.

Studies of familial cancer syndromes, such as retinoblastoma, have lead to the identification of tumor suppressor genes. Loss-of-function mutations in the Rb gene are found not only in all hereditary and sporadic forms of retinoblastoma, but also in many other tumor types, including osteosarcoma, breast carcinoma, small cell lung carcinoma, bladder carcinoma, and prostate carcinoma (1). Introduction of the wild-type Rb gene into Rb-deficient tumor cells suppresses their neoplastic phenotype (2–7), thus establishing the Rb gene as a tumor suppressor.

The Rb gene encodes a nuclear phosphoprotein that undergoes cyclic phosphorylation and dephosphorylation during the cell cycle (8–10). Rb is underphosphorylated during early G1 phase, phosphorylated by members of the cyclin-dependent kinase family just before S phase, and remains phosphorylated until late mitosis. Hypophosphorylated Rb arrests cells in the G1 phase, and phosphorylation relieves this inhibition (11). Rb protein not only plays a major role in the inhibition of G1 to S phase transition (12, 13), but is also important for cell differentiation. In Rb-deficient mouse embryos, sensory neuronal cells fail to become post-mitotic, do not differentiate properly, and exhibit extensive apoptotic cell death. Similarly, lens epithelial cells fail to terminally differentiate and undergo programmed cell death (14, 15).

These pleiotropic cellular functions of Rb are likely to be mediated by association with multiple cellular proteins (16). One of the best studied Rb-associated protein is the transcriptional factor E2F, which regulates expression of several genes essential for S phase entry (17). E2F activity appears to be essential for G1-S progression, since Drosophila E2F null mutants fail to enter S phase after initial 17 embryonic cell divisions (18). The sequestering and concomitant inhibition of E2F by Rb has been used as a paradigm to demonstrate how Rb restrains cell cycle progression. UBF, a ribosomal transcription factor, is another well studied Rb-associated protein (19–21). The UBF-Rb interaction results in suppression of the synthesis of ribosomal RNA by RNA polymerase I, thereby globally inhibiting cell proliferation.

RbAp48 and RbAp46 were first identified as major polypeptides from a HeLa cell lysate that specifically bound to an Rb affinity column (22). The cDNA encoding RbAp48 was subsequently cloned based on partial amino acid sequences. The predicted amino acid sequence of RbAp48 shares significant homology with a known yeast protein, MSI1. MSI1 is presumably a negative regulator of the Ras signal transduction pathway in Saccharomyces cerevisiae, because overexpression of the MSI1 gene suppresses the heat-shock sensitivity of RAS2Val-19 and ira1 mutant yeast strains and reduces the intracellular cAMP levels in these mutants (23). Overexpression of the human RbAp48 gene also suppresses the heat-shock sensitivity of these yeast mutants, suggesting that the yeast and human gene products are functionally homologous (22).

Here we describe the cloning and characterization of RbAp46. RbAp46 and RbAp48 are not only highly homologous at the amino acid sequence level, but also appear to be functionally homologous with each other and with S. cerevisiae MSI1, since overexpression of RbAp46 suppresses the heat-shock sensitivity of RAS2Val-19 mutant yeast strains. We have also isolated mouse cDNAs encoding RbAp48 and RbAp46 and shown that they are evolutionarily conserved.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Analysis of Heat-shock Sensitivity—The relevant genotype of each strain is described in the figure legends. Plasmids pd3 and pd3::48K were described previously (22). Plasmid pYCp48 was constructed by cloning the EcoRI-KpnI fragment of RbAp48 cDNA into the pYC-DE2 vector (24). Plasmid pYCp46 was

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constructed by cloning the coding region of RbAp46 cDNA into the pYC-DE 2 vector. Yeast transformation was done by the lithium acetate method (25). Heat-shock experiments were performed as follows. Fresh transformants were patched onto YPD plates and, after 1 day of growth at 30°C, were replica-plated onto three YPD plates; one of these plates was incubated for 2 days at 30°C, and the remaining two were heat-shocked by incubation at 55°C for 15 or 20 min, respectively, before incubation for 2 days at 30°C.

Construction of COOH-terminal Deletions of GST-RbAp48 and Expression of Fusion Proteins for Epitope Mapping of Antibodies—Plasmid GST-RbAp48 was constructed by cloning the Ncol-KpnI fragment of RbAp48 cDNA into pGEX-2T vector. A series of COOH-terminal deletions of GST-RbAp48 was generated using the procedure developed by Henikoff (26), in which exonuclease III was used to specifically digest insert DNA from a 5’-protruding end. The resulting clones were analyzed by DNA sequencing. Expression of glutathione S-transferase fusion proteins was induced with 0.2 mM IPTG (10 mM). Phages and bacteria were then transferred to nitrocellulose filters saturated with isopropyl-β-D-thiogalactopyranoside (10 mM) and left at 37°C for 4 h. The filters were then incubated with blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 4% bovine serum albumin), then with monoclonal antibody 19H9, followed by alkaline phosphatase-conjugated goat anti-mouse IgG. Antigen-antibody complexes were detected as described above.

Cloning of RbAp46—Using monoclonal antibodies that recognize RbAp46, we screened an expression library for RbAp46 cDNA. From a total of 10\(^6\) phage clones, four clones encoding polypeptides recognized by the monoclonal antibody 19H9 were identified. These clones were analyzed further using monoclonal antibodies 11G10, 12D9, 15B3, and 12B1. Two clones were identified as potential candidates for RbAp46, since the polypeptides were recognized by antibodies 19H9 and 12B1, which react with both RbAp46 and RbAp46, but not by antibodies 11G10, 12D9, and 15B3, which only react with RbAp48. DNA sequencing analysis of these two clones revealed differences with RbAp48 cDNA sequence, whereas the cDNA sequences of the remaining two clones were identical to the RbAp48 cDNA sequence. A full-length cDNA clone was subsequently isolated by re-screening the same library using the partial RbAp46 cDNA as a hybridization probe. DNA sequencing of the RbAp46 cDNA clone revealed a single open reading frame of 425 amino acids with a predicted molecular mass of 47.8 kDa (Fig. 3A). The two RbAp46 polypeptide sequences obtained by protein microsequencing are present within the putative open reading frame. The RbAp46 clone appears to be full-length, because an in-frame stop codon was found upstream of the first putative methionine (underlined DNA sequence).

RESULTS

RbAp46 is Closely Related to RbAp48—RbAp48 and RbAp46 are the two most abundant proteins from HeLa cell lysates specifically retained by an Rb affinity column (22). Analyses of partial peptide sequences indicate that there are sequence similarities between these two polypeptides (see below). Furthermore, mouse polyclonal antibodies raised against the putative RbAp48 recognize both RbAp48 and RbAp46 in the Rb affinity column eluates (Fig. 1). A panel of 13 anti-p48 monoclonal antibodies were also evaluated for their cross-reactivity with RbAp46. Five of them recognize both RbAp48 and RbAp46, whereas the remainder only recognize RbAp48 (Fig. 2B). To further investigate the structural relationship between RbAp48 and RbAp46, we performed immunoblots to identify the regions of RbAp48 recognized by the monoclonal antibodies. A series of COOH-terminal deletions of GST-RbAp48 were generated using the procedure developed by Henikoff (26). A total of 16 COOH-terminal deleted clones were obtained and characterized by DNA sequencing (Fig. 2A). Using this series of mutants, we mapped the epitopes recognized by the 13 anti-p48 monoclonal antibodies (Fig. 2B).

These results indicate that RbAp46 and RbAp48 share sequence homology over their entire lengths, the two polypeptides are unlikely to be a consequence of proteolysis or of differences in post-translational modification.

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Based on the nucleotide sequences, RbAp48 and RbAp46 are most likely unique in the human genome and are encoded by a single-copy gene, whereas RbAp48 either has a very complex genomic structure or shares homology with other genes most likely the products of different genes. To provide further evidence, genomic Southern analysis was performed. Indeed, RbAp46 and RbAp46 display distinct hybridization patterns using the 3′-untranslated regions as probes (Fig. 4). RbAp46 is most likely unique in the human genome and is encoded by a single-copy gene, whereas RbAp48 either has a very complicated genomic structure or shares homology with other genes in the untranslated regions.

Interaction of Rb and RbAp46 in Vivo and in Vitro—We assessed the interaction of RbAp46 with Rb in two ways. First, HeLa cell lysates were immunoprecipitated with anti-Rb antibody 0.495. The immune complexes were analyzed by Western blot with a combination of multiple anti-p48 antibodies recognizing RbAp46. As shown in Fig. 5A, both RbAp48 and RbAp46 were present in the Rb immune complexes. A combination of antibodies was used here, since the monoclonal antibodies have

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Footnote:

1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
very low affinity for RbAp46. Although the combination of these antibodies works well in immunoblotting, they do not recognize native forms of RbAp46, precluding reciprocal immunoprecipitation experiments. Second, a nondenaturing polyacrylamide gel was used to assess the direct interaction among RbAp46, RbAp48, and Rb. Under appropriate conditions, RbAp46, RbAp48, and p56RB (carboxyl terminus of the Rb protein) each migrated as a single band on a nondenaturing polyacrylamide gel (Fig. 5B). When RbAp46 and p56RB were incubated together prior to electrophoretic separation, a new migrating protein band was identified (Fig. 5B, left panel). This new band contains RbAp46-Rb complexes as shown by excision of the band followed by separation of the proteins by SDS-polyacrylamide gel electrophoresis (Fig. 5B, left panel). Similarly, complexes were formed between RbAp48 and Rb (Fig. 5B, left panel) (22). Although RbAp48 and RbAp46 each forms complexes with Rb, they appear not to interact with each other, and no heterodimer is formed when the two proteins are incubated together. In addition, RbAp46 and RbAp48 do not appear to have a synergistic effect on their Rb binding ability (Fig. 5B, left panel).

RbAp46 Suppresses the Heat-shock Sensitivity of the Yeast Strains Containing RAS2Val-19—Since RbAp48 can suppress the heat-shock sensitivity of RAS2Val-19 mutant yeast strains, we tested whether RbAp46 can also suppress the heat-shock sensitivity of the same yeast strains. Introduction of either pd3, the yeast MSI1-containing plasmid, or pd3::48k, the plasmid expressing RbAp48 from the yeast MSI1 promoter, or pYCp48, the plasmid expressing RbAp48 from the strong yeast ADH1 promoter, or pYCp46, the plasmid expressing RbAp46 from the ADH1 promoter, but not the control plasmid pYC-DE2, conferred resistance to heat shock at 55°C for 15 min. Only pd3 transformants can survive heat shock at 55°C up to 20 min (Fig. 6). Expression of RbAp48 or RbAp46 in the transformants was confirmed by immunoblotting (data not shown). Although the mechanism of heat-shock resistance is unknown, these data provide evidence that RbAp48 and RbAp46 both mimic the function of MSI1 in yeast.

Isolation of Mouse RbAp48 and RbAp46 cDNA—Mouse RbAp48 (mRbAp48) cDNA was isolated by screening a mouse brain cDNA library using human RbAp48 cDNA as a hybridization probe. DNA sequencing of the mRbAp48 cDNA clone revealed a single open reading frame of 425 amino acids. Like human and mouse RbAp48, the putative human and mouse RbAp46 amino acid sequences are identical except for one conserved amino acid substitution (Fig. 7). These results indicate that RbAp48 and RbAp46 are highly conserved during evolution and may have distinctive functions.
Fig. 3. Sequence of RbAp46 and sequence comparison. A, cDNA sequence and deduced amino acid sequence of RbAp46. The numbers correspond to the nucleotide (upper line in each pair) or amino acid (lower line in each pair). An open reading frame of 425 amino acid residues is shown. Peptide sequences identical to that obtained from purified protein are underlined. B, comparison of aligned amino acid sequences of RbAp48 and RbAp46. The complete sequences of the two proteins are shown. Identical amino acids are shown by the solid vertical line; conservative amino acid substitutions are shown by the colons (these amino acids are grouped as ILMV, AS, TS, KR, DE, DN, QN, and QE). The dashes indicate the placement of gaps to maximize alignment between the two sequences.
Expression of RbAp48 and RbAp46—RNase protection analysis was carried out to examine the expression of both RbAp46 and RbAp48 mRNA in different mouse tissues. Although both RbAp48 and RbAp46 mRNA were detected in all of the mouse tissues tested, their mRNA levels vary dramatically between different tissues (Fig. 8). Higher levels of RbAp48 and RbAp46 mRNA were observed in brain, thymus, lung, spleen, kidney, testis, and ovary/uterus tissues; and lower levels were observed in heart, liver, and muscle tissues. The levels of RbAp46 and RbAp48 were similar in most of the mouse tissues except testis and thymus, in which RbAp48 was higher than RbAp46, and ovary/uterus, in which RbAp46 was higher than RbAp48. These results indicate that expression of both RbAp46 and RbAp48 is regulated in a tissue-specific manner.

DISCUSSION

In this study, we have cloned and characterized RbAp46, an Rb-associated protein which shares high homology (89.4% identity) with the previously characterized RbAp48 (22). The RbAp48 and RbAp46 polypeptides share many common features; both are nuclear proteins and bind Rb protein in vitro and in vivo; both contain the same internal WD (Trp-Asp) repeats; both share amino acid sequence homology with yeast protein MSI1 and mimic the function of MSI1 in yeast; both share similar tissue expression patterns, except testis and thymus, in which RbAp48 was higher than RbAp46, and ovary/uterus, in which RbAp46 was higher than RbAp48. These results indicate that expression of both RbAp46 and RbAp48 is regulated in a tissue-specific manner.

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RbAp48 and RbAp46 are evolutionarily conserved. The predicted amino acid sequences of RbAp48 for human and mouse are completely identical, and the predicted protein sequences of RbAp46 for human and mouse are identical, except for one conserved amino acid substitution. Homologs of RbAp48 and RbAp46 have been cloned from tomato and Arabidopsis thaliana as well, sharing 65% amino acid sequence identity to the human counterparts. The high degree of conservation of RbAp48 and RbAp46 genes during the course of evolution is indicative of their functional importance.

The presence of WD repeats in both RbAp48 and RbAp46 is of interest. The WD repeat was first found in the β-subunit of heterotrimeric GTP-binding proteins (G proteins) which transduce signals across the plasma membrane (29). It has been called the β-transducin repeat (30), the WD-40 repeat (29, 31), or the GH-WD repeat (32). WD repeat proteins are made up of highly conserved repeating units usually ending with Trp-Asp (WD) and have been found in all eukaryotes but not in prokaryotes. These proteins appear to perform regulatory functions in diverse cellular processes, such as cell division, cell fate determination, gene transcription, transmembrane signaling,
mRNA modification, and vesicle fusion (33). In mammalian cells, only a few nuclear WD repeat proteins have been characterized. Recently, the p60 subunit of chromatin assembly factor I has been cloned and shown to contain WD repeats (34). However, it is unclear whether the WD repeats are required for chromatin assembly factor I function. On the other hand, several WD proteins have been shown to form multiprotein complexes, interacting with other proteins through the WD repeat region (31, 35–38). Preliminary data indicate that the WD repeat region of RbAp48 is not required for its interaction with Rb.\(^3\) It will be of interest to test whether, besides Rb, RbAp48 interacts with other cellular proteins.

Although homologs of RbAp48 and RbAp46 genes have been found in plants and animals, it is not clear whether yeast has other genes homologous to MSI1. Gene disruption experiments indicated that the MSI1 gene is not essential for the growth of yeast cells. Yeast cells carrying the MSI1 null mutation were indistinguishable from wild-type cells with regard to growth rate, sporulation efficiency, and heat-shock sensitivity (23). It is possible that there is a second gene in yeast that can complement at least some of the functions of MSI1. Although it has been speculated that MSI1 negatively regulates Ras activities, nothing is known about the mechanisms involved (23). The central role of Ras proteins in control and differentiation in many species and, specifically, in neoplasia in humans has been well documented (39–41). Many aspects of Ras signaling are similar in yeasts and mammals. Mammalian RAS genes are functional in yeast, and mutated yeast RAS genes efficiently transform mouse fibroblast cell lines. Although in lower and higher eukaryotes, Ras regulates different biochemical pathways, Raf1 and mitogen-activated protein kinase in mammals (42) and adenylyl cyclase in yeasts (43, 44), there is some evidence indicating that the effects of Ras activity on gene expression are similar in yeasts and in mammals. For example, both in yeasts and in mammals, Ras activates AP-1 transcription factors in response to UV irradiation (45) and controls transcription of similar heat-shock genes in response to heat shock (46). It could be that many effects of Ras on gene expression are crucial for cellular responses to external signals and, therefore, are highly conserved in evolution. However, the pathways that transmit the signal from Ras to the transcription factors are not conserved. Since both RbAp48 and RbAp46 are functionally equivalent to MSI1 in the suppression of the Ras mutant phenotype, it could be that RbAp48 and RbAp46 in mammals and MSI1 in yeasts regulate similar effects of Ras on gene expression in response to external signals. The high degree of conservation of RbAp48 and RbAp46 genes during the evolution of different species suggests that they play important roles in cellular processes.

\(^3\) Y.-W. Qian and E. Y.-H. P. Lee, unpublished results.

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**Fig. 7.** Sequencing comparison of RbAp48 and RbAp46 between human and mouse. The predicted amino acid sequence of human RbAp48 is shown. Amino acid residues of mouse RbAp48 and human and mouse RbAp46 that are identical to those of human RbAp48 are shown by asterisks. Gaps (dashed lines) were inserted to optimize the alignment.

**Fig. 8.** Expression of RbAp46 and RbAp48 genes in mouse tissues. A, a 10-μg sample of total RNA from 2-month-old mouse tissues was subjected to RNase protection assays to detect the presence of RbAp48 and RbAp46 mRNA. B, the same amount (10 μg) of total RNA samples was run on a 1.2% formaldehyde agarose gel and visualized by ethidium bromide staining. C, the abundance of RbAp48 and RbAp46 mRNA in A was densitometrically quantitated and normalized to the 28S and 18S rRNAs. The amount of RbAp48 in the brain was arbitrarily adjusted to 1. The RNase protection experiment was repeated three times with similar results.
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