Cloning and Characterization of a Novel Kruppel-associated Box Family Transcriptional Repressor That Interacts with the Retinoblastoma Gene Product, RB*

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The retinoblastoma gene product, RB, seems to function as a key tumor suppressor by repressing the expression of genes activated by members of the E2F family of transcription factors. In order to accomplish this, RB has been proposed to interact with a transcriptional repressor. However, no genuine transcriptional repressors have been identified by virtue of interaction with RB. By using the yeast two-hybrid system, we have identified a novel member of a known family of transcriptional repressors that contain zinc fingers of the Kruppel type and a portable transcriptional repressor motif known as the Kruppel-associated box (KRAB). The mouse and human forms of the novel RB-associated KRAB protein (RBaK) are widely expressed. The amino acid motif that links the KRAB domain and zinc fingers appears to be required for interaction with RB in vitro. Human RBaK ectopically expressed in fibroblasts is an 80-kDa protein that is localized to the nucleus. The expression of either RB or RBaK in 10T1/2 fibroblasts represses the activation of an E2F-dependent promoter and decreases DNA synthesis to a similar degree. However, a mutant form of RBaK that cannot interact with RB in vitro is unable to prevent DNA synthesis. We present a model in which RB physically interacts with the novel transcriptional repressor RBaK to repress E2F-dependent genes and prevent DNA synthesis.

Over the last decade, many studies of cancer susceptibility genes have yielded new insight into both the pathogenesis of malignancy and the normal biology of cellular and developmental processes. The retinoblastoma gene, RB, provides an elegant example. The RB gene was initially discovered by positional cloning strategies and shown to be mutated in patients with hereditary retinoblastoma; hence, RB was the first bona fide tumor suppressor gene (reviewed in Refs. 1 and 2). Much subsequent work has focused on the molecular mechanism by which its gene product, RB, controls key aspects of cellular proliferation and the mechanism by which this function is regulated.

Initial studies indicated that one normal function of RB1 is to arrest cell proliferation by preventing the entry of cells into S phase of the cell cycle. When the RB protein is delivered to cells by microinjection of protein or transfection of cDNA, it suppresses the malignant phenotype (3) and blocks entry of cells into S phase (4–7). The ability of RB to prevent cell growth depends on its functional domains, termed the A and B motifs, and on its carboxyl terminus (5). The A and B motifs are bound and presumably inactivated by several viral oncoproteins such as simian virus 40 large T antigen and adenovirus E1a oncoprotein (8–12). These motifs are also the sites of many native cancer-causing mutations in the RB gene (11, 12). Together, the carboxyl terminus and the A and B motifs of RB have been shown to bind to a number of cellular proteins as well as to the viral oncoproteins noted above (1). RB is thought to accomplish its diverse cellular functions, including its tumor suppressor activity, by interacting with these cellular proteins. Perhaps chief among the growing list of proteins known to interact with RB is E2F-1 (13–17).

E2F-1 was the first identified member of a family of transcriptional activators (collectively known as E2Fs) that includes at least six E2Fs and two heterodimeric DNA-binding partners, DP-1 and -2 (reviewed in Refs. 18–20). E2F-1 binds to elements of DNA that mediate cell cycle-dependent induction of a number of gene products as cells initiate DNA synthesis such as dihydrofolate reductase (21), b-Myc (22, 23), c-Myc (24), and E2F-1 itself (14, 25, 26). The importance of RB binding to E2F-1 was suggested by the following findings: 1) naturally occurring mutations in the A and B motifs of RB prevent RB-E2F interaction (27–29); 2) the physical interaction of RB with E2F-1 correlates with the transcriptional repression of genes thought to be activated by “free” E2F-1 (24, 30, 31); and 3) the ectopic expression of E2F-1 can drive cells into S phase (32–34) and overcome RB-induced growth arrest (29, 35). Together, these findings all support a model in which RB functions as a tumor suppressor by physically interacting with E2Fs to repress the
expression of E2F-dependent genes.

Two general issues can be raised regarding possible mechanisms by which RB may repress transcription. First, it is not clear whether RB has an intrinsic capacity to repress gene expression or whether RB accomplishes this repression by associating with additional proteins such as a transcriptional repressor. Second, it is not known whether RB directly affects the function of the transcriptional apparatus or secondarily regulates the transcriptional machinery by regulating histone deacetylase activity. With respect to the former issue, intrinsic RB repressor activity seems paradoxical to the documented ability of RB to enhance the activity of other transcription factors. For example, the transactivation function of MyoD (27, 36, 37), C/EBP (38), NF-IL6 (39), GAL4-Myc (40), and ATF-2 (41) may be enhanced by direct interaction with RB. With respect to the latter issue, although recent findings suggest that RB may affect chromatin structure by regulating histone deacetylase activity, this may not quantitatively account for all RB-mediated repression and may apply only to a specific subset of genes that can be repressed by RB (42, 43). Hence, although there may be multiple mechanisms for RB-mediated transcriptional repression, we favor a model in which RB can repress E2F-dependent genes by forming a multimeric complex with E2F-1 and a putative transcriptional repressor. Such a model has been previously proposed (44), and one transcriptional repressor, HBP1, has been shown to interact with RB and the RB-like protein p130 (45). However, the functional importance of HBP1 with respect to RB function to arrest cell proliferation has not been established.

Because RB is essential for the development of the murine nervous system (reviewed in Ref. 46), we sought to identify RB-interacting proteins that are expressed in the mouse embryonic brain. As part of this effort, we have identified a novel member of a known family of transcriptional repressors. This family of genes contains zinc fingers of the Kruppel-type (47, 48) and a portable transcriptional repressor motif known as the Kruppel-associated box (KRAB) (49). Our studies indicate that this RB-associated KRAB (RBaK) protein can physically interact with RB and may contribute to RB-dependent suppression of E2F-mediated transcriptional activation and RB-mediated cell cycle arrest.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Subcloning Strategies**—Plasmids used in the yeast two-hybrid screen included pAS1-RB (p56) (amino acids 295–928 of mouse RB), pAS1-RB(A) (deletion of amino acids 434–476), and pAS1-RB(B) (deletion of amino acids 697–763). All of these were created by subcloning the described sequences into the pAS1 plasmid (50). We used pAS1-RB(p56), which encompasses amino acids 295–925 of mouse RB, as the “bait” to screen a 1.5 × 10^6 λ phage cDNA library. This λ ACT cDNA library, like one previously described (50), was generated from RNA isolated from the heads of mouse E13.5 embryos and the brains of newborn mice (provided by E. Olson). The resulting cDNAs were fused with the GAL4-activation domain and driven by the alcohol dehydrogenase (ADH) promoter (50). Plasmids from β-galactosidase-positive colonies that formed in the presence of pAS1-RB(p56) but not in the presence of mutated forms of RB were analyzed further.

**Cloning of Mouse and Human RBaK**—Larger forms of mouse and human RBaK cDNA were sought by screening other cDNA libraries as follows. A cDNA probe was made by using PCR to amplify a 388-bp fragment from pACT-mM17.1 that corresponds to amino acids 126–255 of mouse RBaK (Fig. 1B) (5′ primer, 5′-tctggtaacagggagatttt-3′; 3′ primer, 5′-cattctactcagatcggcagt-3′). This PCR product was gel-purified, labeled with [γ-32P]dCTP as described above, and hybridized to the RNA blot using a cDNA probe generated by PCR to amplify the coding sequence of hRBaK from bp 523 to 1032, which corresponds to the protein sequence from the second amino acid through the first half of the linker motif (Fig. 1A). The 5′ primer encoded an artificial nuclear localization signal (MAPKKKKKRR) adjacent to the RBaK-specific sequence. EcoRI and XbaI sites were added to the 5′ and 3′ primers, respectively. This amplified PCR product was subcloned, in frame, to the EcoRI-XbaI sites in pCS2-MTS.

**Yeast Two-hybrid Screen**—The yeast two-hybrid screen to isolate cDNA encoding RB-interacting proteins was performed essentially as described previously (50). We used pAS1-RB(p56), which encompasses amino acids 295–925 of mouse RB, as the “bait” to screen a 1.5 × 10^6 λ phage cDNA library. This λ ACT cDNA library, like one previously described (50), was generated from RNA isolated from the heads of mouse E13.5 embryos and the brains of newborn mice (provided by E. Olson). The resulting cDNAs were fused with the GAL4-activation domain and driven by the alcohol dehydrogenase (ADH) promoter (50). Plasmids from β-galactosidase-positive colonies that formed in the presence of pAS1-RB(p56) but not in the presence of mutated forms of RB were analyzed further.
The 5'- primer for human RBaK-KL and -KDL was 5'-tcattcaaa-
gatgtggctgtgga. The 3'- primers were 5'-caaagggcttcatttccatttgtg and 5'-gttttcccacattcattacactca for human RBaK-KL and -KDL, respectively. The proteins encoded by these PCR products include amino acids 9–259 and 9–169 for mouse RBaK-KL and -KDL, respectively, and amino acids 9–261 and 9–170 for human RBaK-KL and -KDL, respectively. To generate hRBaK-LZ, which encoded a protein from amino acids 80 to 364, we used the 5' and 3' primers 5'-gaagcctggagagttgat-
gacc and 5'-tggtgcagggtgagatgtgtc, respectively. The 5' primers for all of these PCR reactions contained the T7 RNA polymerase and translation initiation signals 5'-gctaaatacgactcactataggaacagaccaccATG-3' joined to the RBaK-specific sequences.

For the GST pull-down assay, equivalent amounts of in vitro translated proteins were incubated with GST or GST-RB(p56) bound to glutathione-Sepharose beads equilibrated in EBC buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) with 4% bovine serum albumin and 1 mM phenylmethylsulfonyl fluoride, for 90 min at 4 °C. The glutathione beads were then washed 4 times with EBC buffer, and the bound proteins were solubilized in SDS-gel loading buffer, fractionated by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography.

**Fig 1.** Human and mouse RBaK cDNA encode related proteins containing the KRAB motif and Kruppel-type zinc fingers. A, partial cDNA and predicted amino acid sequence for human RBaK. The first ATG sequence (bp 519), KRAB A and B motifs (bp 537–756), GEKPY links between Cys2 and His2 zinc fingers and stop codon are underlined. Note that the link between the 8th and 9th Cys 2 and His2 zinc fingers is degenerate (double underline). B, alignment of predicted amino acid sequence for mouse and human RBaK. Nucleotides in the human sequence that are identical in mouse are denoted by a dot. KRAB A and B motifs are shaded. Cysteine and histidine residues defining reiterated zinc fingers are in boldface type. GEKPY His2 and Cys2 links are underlined. Note that the GEKPY link between the 8th and 9th zinc fingers is degenerate in both mouse and human proteins and that there is a GEKPY sequence in both mouse and human protein that is not flanked by zinc fingers (double underlined). C, schematic diagram of RBaK showing KRAB, linker, and zinc fingers (ZF) and the percentage of amino acid identity between mouse and human RBaK.
activity by using Galacto-Light Plus (Tropix). CAT activity from several representative experiments, each with duplicate samples, was normalized to the $\beta$-galactosidase level and expressed as the fold increase over the activity from cells transfected with no reporter plasmid.

**Protein Expression and Cell Proliferation Assays**—To analyze the size and subcellular localization of ectopically expressed hRBaK, we transfected pCMV-RBaK, pCMV-MT6hRBaK, or pCMV-MT6hRBaK-KD into 10T1/2 fibroblasts by using LipofectAMINE (Life Technologies, Inc.). Cells were maintained in growth medium for 48 h after transfection and then were harvested in Nonidet P-40 lysis buffer as described previously (52) or fixed by using 2% paraformaldehyde. Ectopically expressed human RBaK and human Myc-RBaK were detected by using either mouse anti-RBaK antiserum with alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development or mouse 9E10 anti-Myc antibody with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and enhanced chemiluminescence. For immunofluorescence staining, Myc-tagged proteins were detected in paraformaldehyde-fixed cells using mouse 9E10 anti-Myc antibody and rhodamine- or fluorescein-conjugated secondary antibody (Jackson ImmunoResearch), essentially as described previously (36).

To analyze whether the expression of RBaK affected DNA synthesis, 10T1/2 fibroblasts were transiently transfected as described above. In these experiments, cells were co-transfected with pCMV-MT6RBaK, pCMV-MT6RBaK-KD, or pCMV-MT6hRBaK-KL into 10T1/2 fibroblasts by using LipofectAMINE (Life Technologies, Inc.). Cells were maintained in growth medium for 48 h after transfection and then were harvested in Nonidet P-40 lysis buffer as described previously (52) or fixed by using 2% paraformaldehyde. Ectopically expressed human RBaK and human Myc-RBaK were detected by using either mouse anti-RBaK antiserum with alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development or mouse 9E10 anti-Myc antibody with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and enhanced chemiluminescence. For immunofluorescence staining, Myc-tagged proteins were detected in paraformaldehyde-fixed cells using mouse 9E10 anti-Myc antibody and rhodamine- or fluorescein-conjugated secondary antibody (Jackson ImmunoResearch), essentially as described previously (36).

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nus did not (data not shown). Restriction enzyme and sequence analysis of pACT-M17.1 indicated that it contained a 1.4-kb cDNA that was predicted to encode a novel protein with Kruppel-type zinc fingers and a KRAB repressor motif.

Kruppel-type zinc fingers (56) are tandemly repeated motifs containing two cysteine residues and two histidine residues (Cys2-His2). These zinc fingers are typically separated by the conserved amino acid sequence XGKEPXY (single letter amino acid designation) (47, 48). Approximately one-third of the estimated several hundred mouse or human genes containing these Kruppel-type zinc fingers are thought to also contain a KRAB motif (49, 57), which harbors potent transcriptional repression activity (58–61). No KRAB protein has previously been shown to interact with RB or RB-like proteins. Hence, we chose the term RBaK for this novel cDNA, which apparently encodes an RB-associated KRAB protein, and we performed further studies to begin to characterize its function.

**Sequence Analysis of Mouse and Human RBaK—**Because pACT-mM17.1 appeared to contain a partial cDNA, we screened mouse embryo (E11.5) and human HeLa cell cDNA libraries for longer forms. We used the 388-bp PCR product encoding the “linker” motif (amino acids 126–255) between the KRAB domain and zinc fingers to isolate two additional overlapping mouse cDNA clones and one human cDNA clone. Sequence analysis of the longest human cDNA clone (hM17.6) indicated that it contains a 2142-bp open reading frame (Fig. 1A). The proposed coding sequence is preceded by 518 bp of apparently untranslated sequence before the first ATG, which conforms roughly to a Kozak consensus sequence (A in position −3, A in position +4) (62) and is preceded by an in-frame stop codon 188 bp 5′ of the ATG codon (Fig. 1A). The putative translation initiation codon and in-frame stop codon were observed in an additional human cDNA clone that was subsequently isolated using hRBaK cDNA as a probe (data not shown). The 2448-bp sequence 3′ to the stop codon contains a polyadenylation signal and a poly(A) tail. A search of published DNA data bases for sequences similar to mouse and human RBaK demonstrated that they had various degrees of similarity to numerous previously identified proteins containing KRAB motifs and Kruppel-type zinc fingers but no identical match. Thus, human and mouse RBaK appear to be novel members of the KRAB/zinc finger family.

Predicted amino acid sequences of both mouse and human RBaK indicated that they contain KRAB A and B motifs (49) at their amino termini. Although many KRAB proteins have both of these motifs, most of the transcriptional repressor activity of these proteins seems to reside in the A motif (58–61, 63). At the carboxyl terminus of the predicted RBaK protein, there are 15 (mouse) and 16 (human) tandemly repeated Kruppel-type zinc fingers (Fig. 1B). In mouse and human RBaK, the GKEKPY sequence after the 8th zinc finger and the H2 component of the 10th zinc finger are degenerate. In mouse RBaK, an apparent stop codon is found to interrupt what would be the 16th zinc finger (Fig. 1B). Such an abrupt stop is unusual among members of the KRAB family (60, 64, 65), suggesting that this stop codon may not represent the true 3′ end of the coding sequence for mouse RBaK. In both mouse and human RBaK, a linker motif of approximately 180 amino acids separates the KRAB domain and the zinc fingers (Fig. 1, A and B). Interestingly, both also contain a single GKEKPY amino acid sequence, which is not flanked by zinc fingers, in the linker 29 residues amino-terminal to the first zinc finger (Fig. 1B). Although DNA sequence within this linker region is thought to define subfamilies of KRAB-containing proteins (65, 66), the DNA sequence of the mouse and human RBaK linker was not similar to that of other published KRAB family genes.

Overall mouse RBaK and human RBaK are 78% identical at the amino acid level. There is 84 and 90% identity in the KRAB and zinc finger motifs, respectively, and 54% identity in the linker region (Fig. 1, B and C). Interestingly, within the mouse and human RBaK linker motifs, there is more identity in the carboxyl-terminal region, which we have shown to be required for RB interaction in vitro (see below), than in the amino-terminal linker motif. In summary, on the basis of DNA and predicted amino acid sequence, mouse and human RBaK seem to be novel, potentially orthologous members of the KRAB family of transcriptional repressors.

**Mouse and Human RBaK Are Widely Expressed in Embryonic and Adult Tissues—**Because RBaK was cloned from a neuron-enriched cDNA library, we wanted to determine its expression pattern using Northern blotting. We anticipated that this might provide insight as to whether RBaK might have cell type-specific effects.

Because the KRAB gene family is large, we used a cDNA probe from the 5′-untranslated region of the human RBaK cDNA for hybridization with a commercially available Northern blot containing RNA from a variety of human tissues. This probe detected one approximately 5.5-kb transcript in all tissues examined (Fig. 2). Similar wide expression of RBaK was found in mouse tissues by hybridization of the mouse RBaK with Northern blots containing mouse mRNA (data not shown). Although the size of the human RBaK transcript corresponded approximately to the length of the cloned cDNA, it is not clear whether the cloned cDNA included the full 5′-untranslated region. We were unable to extend this cDNA to the 5′ by using rapid amplification of cDNA ends technology. However, this approach may have been complicated by the GC-rich nature of the 5′ end of the cDNA. When Northern blots were hybridized with a probe encoding the linker region of hRBaK, an approximately 8-kb transcript and a larger (7.1-kb) transcript were detected (data not shown). The larger transcript may represent either a related gene product or different splicing of the hRBaK transcript, an occurrence that has been described for other members of this gene family (61, 67–70). The wide expression of human and mouse RBaK suggests that it may not have cell type-specific functions and that it may contribute to general effects of RB on cell growth arrest rather than to the lineage-dependent effects of RB on cellular differentiation.

**A Conserved Linker Is Required for Interaction of Mouse and Human RBaK with GST-RB—**Despite the sequence similarity between mouse and human RBaK, the large size of the KRAB gene family prompted us to use an in vitro interaction assay to confirm that human RBaK interacted with RB. The human RBaK cDNA was transcribed and translated in vitro and ap-
plied to GST or GST-RBp56. The mass of the translated product was ~80 kDa (predicted mass, 78.5 kDa) that was consistent with translation from the first ATG (at bp 519) in the cDNA (Fig. 3A). Human RBAK that was translated in vitro physically interacted with GST-RB but not with GST alone (G). B, autoradiograph of [35S]methionine-labeled forms of mouse RBAK and human RBAK that were transcribed and translated in vitro, incubated with GST (G) or GST-RB (RB)-Sepharose beads, and analyzed by SDS-polyacrylamide gel electrophoresis. Input represents one-tenth of the volume of reticulocyte lysate incubated with GST protein. C, schematic diagram showing mutated forms of RBAK used in A and B.

Because there are no known RB-interacting motifs in RBAK proteins, we next sought to determine which motifs of mouse and human RBAK are required for RB binding. In order to determine which defined region of RBAK (the KRAB motif, zinc fingers, or linker region) was required for binding to RB, we performed a series of in vitro binding experiments using different forms of RBAK. Different lengths of the mouse and human RBAK were transcribed and translated in vitro to generate proteins containing the KRAB and linker (KL), the KRAB, and the first half of the linker (KΔL), or the linker region and several zinc fingers (LZ) (Fig. 3B, lanes 1–4 and 13). These translated proteins were then assayed for their relative ability to bind to GST or GST-RB in vitro. Like full-length human RBAK (Fig. 3A), both mouse and human RBAK-KL bound well to GST-RB but not to GST alone (Fig. 3B, lanes 5, 6, 9, and 10); therefore, the zinc fingers were not required for binding to GST-RB in this assay. However, when the carboxyl-terminal half of the linker region was removed, the binding of both mouse and human RBAK-KΔL to GST-RB was greatly reduced (Fig. 3B, compare lanes 8 and 12 with lanes 6 and 10), a finding indicating that an intact linker motif is required for both mouse and human RBAK-KL binding to GST-RB. Finally, in vitro translation of the linker and several zinc fingers from human RBAK (hRBAK-LZ) yielded a peptide that bound GST-RB with an affinity approximating that of RBAK forms retaining the KRAB domain (Fig. 3B, lanes 13–15; some data not shown). Together, these results indicate that neither the KRAB motif nor the zinc fingers are required for GST-RB binding in this assay, whereas an intact linker motif may be required (Fig. 3C).

**Human RBAK Is a Nuclear Protein of Approximately 80 kDa**—Having confirmed that both mouse and human forms of RBAK can interact with GST-RB in vitro and in the yeast two-hybrid system, we next began to characterize the functional properties of RBAK in vivo. After a plasmid containing the CMV promoter driving the expression of hRBAK was transfected into mouse 10T1/2 fibroblasts, mouse antiserum to human RBAK detected the expression of an ~80-kDa protein. When cells were transfected with mutated RBAK cDNA (which
generates Myc-tagged RBaK-KL), the anti-hRBaK antisera detected a 55-kDa protein instead of the 80-kDa protein (Fig. 4A, lanes 1–3). Neither protein was detected in vehicle-transfected cells (data not shown). The 80-kDa protein detected in the presence of full-length RBaK cDNA was similar in size to the protein obtained by using in vitro transcription and translation (Fig. 3A), a finding consistent with the open reading frame predicted by the sequence analysis. The additional 85-kDa protein present in every lane may be an endogenous protein that is detected nonspecifically (Fig. 4A).

Having confirmed that human RBaK could be ectopically expressed, we next sought to determine its subcellular localization using a Myc epitope-tagged form of human RBaK. In vehicle-transfected cells, anti-Myc antibody produced no detectable staining (data not shown). However, in cells transfected with pCMV-MT6RBaK, there was staining diffusely within the nucleus and apparently excluded from nucleoli (Fig. 4B). This subcellular distribution is consistent with that of a previously reported, apparently unrelated KRAB-containing protein ZNF74 (71), whose nuclear localization was shown to be dependent on the zinc finger motifs. The nuclear localization of RBaK is consistent with its proposed role as a transcriptional regulator and with its potential physiological interaction with RB.

RBaK Can Repress E2F-dependent Genes in a DNA Binding-dependent Manner—Heterologous fusion proteins containing a DNA binding domain and a KRAB motif from other proteins have been shown to repress transcription in a DNA binding-dependent manner (58–61, 63). We observed that the protein encoded by the pACT-M17.1 insert had transcriptional repressor activity when fused to a GAL4 DNA binding domain (data not shown), a finding that was consistent with the previous observations. Next, we wanted to determine whether RBaK could function as a transcriptional repressor without being fused to a heterologous DNA binding motif. Because RB seems to function as a tumor suppressor by repressing the expression of E2F-1-dependent genes, we investigated whether the forced expression of RBaK would also repress an E2F-dependent promoter.

We co-transfected 10T1/2 fibroblasts with different CAT reporter plasmids with an expression plasmid encoding Myc-tagged human RBaK or RB or the empty vehicle plasmids. The expression of reporter plasmids containing the thymidine kinase promoter and either two wild-type E2F-1 DNA-binding sites or two mutated E2F-1-binding sites induced the expres-
sion of CAT enzyme by 38- and 12-fold, respectively, relative to vehicle-transfected cells (Fig. 5A, lanes 1, 2, 5, and 6). The expression of this latter reporter is likely driven by other elements in the promoter that are independent of E2Fs. Under these conditions, the expression of either human RB or RBaK significantly repressed the E2F-dependent activation of E2F(wt)CAT (Fig. 5A, lanes 2–4). In these experiments, the repression by RB could account for nearly 100% of the E2F-dependent activity (Fig. 5A, lanes 2, 4, and 6), whereas repression by RBaK could account for ~70% of this E2F-dependent activity (Fig. 5A, lanes 2, 3, and 6). Although there were quantitative differences in the magnitude of repression by RB and RBaK, it was qualitatively the same because neither protein repressed E2F-independent promoter activity (Fig. 5A, lanes 6–8). We have not consistently observed cooperative repression in 10T1/2 cells ectopically expressing RB or RBaK. However, under the conditions we tested, the expression of RB alone nearly completely repressed the E2F-dependent activation of this reporter. This finding would make it impossible to see a cooperative interaction between RB and RBaK. Further studies using limiting dilutions of each plasmid will be required to determine whether there are cooperative interactions between RB and RBaK in this assay.

In parallel assays, cells were also transfected with a CAT reporter plasmid containing the constitutive CMV promoter, which leads to greater than 250-fold induction of CAT enzyme activity (Fig. 5B, lanes 1 and 2). The expression CMV-CAT was minimally repressed by either co-transfected RB or RBaK (Fig. 5B, lanes 2–4). Therefore, the absence of repression of the E2F(wt)CAT reporter by either RB or RBaK is not simply due to the lack of high activation of the E2F(wt)CAT reporter. Taken together, these results are consistent with a model in which RB and RBaK both repress the expression of a promoter that may be activated by E2Fs, but they do not appear to have general transcriptional repressor effects on other promoters.

Ectopically Expressed RBaK and RB Similarly Decrease BrdUrd Incorporation in 10T1/2 Fibroblasts—Because RB and RBaK can similarly repress the expression of an E2F-dependent promoter, we wanted to determine whether they could also prevent the entry of 10T1/2 cells into S phase of the cell cycle. To address this, we chose to determine whether the transient transfection of these cells with expression plasmids encoding either RBaK or RB would block these cells from incorporating BrdUrd, a marker of DNA synthesis, which could be detected by immunofluorescence staining (Fig. 6). In these experiments the cells were co-transfected with one of these expression plasmids as well as an expression plasmid encoding the β-galactosidase gene. The ectopic β-galactosidase protein, detected by immunofluorescence staining, was used to mark the transfected cells (Fig. 6). After transfection, the cells were grown in medium containing BrdUrd and were then processed for immunofluorescence analysis. The transfected cells (cells that were positive for β-galactosidase) were scored as being BrdUrd-positive or BrdUrd-negative (Fig. 6) and were counted to determine whether transfection with RB or RBaK decreased the incorporation of BrdUrd into the cells.

By determining the proportion of transfected cells that are BrdUrd-positive or -negative, we demonstrated that the ectopic expression of either RB or RBaK increased the number of BrdUrd-negative cells by almost 10% (Table I). The magnitude of the effect of RB likely reflects the ability of these particular cells to overcome partially the growth arrest induced by RB. It is quite interesting, then, that RBaK and RB had quantitatively similar effects in this assay because we would propose that they may be functioning together. Hence, it is conceivable that the same cellular mechanisms to overcome RB-induced growth arrest may also overcome RBaK-induced growth arrest.

To explore further whether RBaK and RB may be functioning together to decrease BrdUrd incorporation in these cells, we wanted to determine whether a form of RBaK that cannot form a complex with RB to prevent DNA synthesis would still alter BrdUrd incorporation. To do this, we performed a similar series of experiments using a mutated form of RBaK that contained only the KRAB motif and the first half of the Linker (RBaK-KΔL). We had previously shown that RBaK-KΔL seemed to be largely unable to bind to RB in vitro as compared with forms of RBaK that retain the full Linker motif and zinc fingers (Fig. 3, A and B). Therefore, if RBaK were preventing BrdUrd incorporation by forming a repressor complex with RB, RBaK-KΔL should not be able to decrease BrdUrd incorporation.

When these cells were co-transfected with a plasmid encoding RBaK-KΔL and β-galactosidase as described above, RBaK-KΔL did not decrease the incorporation of BrdUrd (Table I). This finding is consistent with a proposed mechanistic model in which RBaK forms a complex with RB to prevent DNA synthe-

![Figure 6](image.png)

**TABLE I**

| Transfected plasmid | # BrdUrd + cells (%) | # BrdUrd− cells (%) |
|---------------------|----------------------|---------------------|
| Vehicle             | 614 (33.4, 31.2–35.6) | 1224 (66.6, 64.4–68.8) |
| RB                  | 239 (23.7, 21.2–26.5) | 767 (76.3, 73.5–78.8) |
| RBaK                | 421 (25.9, 23.9–27.2) | 1362 (75.0, 72.8–77.7) |
| RBaK-KΔL            | 334 (33.4, 30.5–36.4) | 666 (66.6, 63.6–69.5) |

*The decrease in the number of BrdUrd-positive-transfected cells in the presence of RB or RBaK was statistically significant as compared with vehicle-transfected cells (p < 0.001, Fisher's exact test).
**FIG. 7.** Ectopic Myc-tagged hRBaK-KΔL is localized to the nucleus and expressed to high levels. A, a photomicrograph depicting immunofluorescence staining of 10T1/2 cells transfected with expression plasmid encoding Myc-tagged hRBaK-KΔL. Cells were stained with anti-Myc epitope antibody. B, Western blot of whole-cell lysates of 10T1/2 fibroblasts transfected with expression plasmid encoding Myc-tagged human RBaK-KΔL (lanes 2 and 3) or RBaK (lane 4). Lanes 2 and 3 represent cells transfected with two separate preparations of the MT-RBaK-KΔL expression plasmid.

sis. However, because RBaK-KΔL is a mutated form of RBaK, its activity could also be altered because it may generate an unstable subcellular compartment (i.e. the nucleus). Therefore, as a control for these possibilities, we confirmed that ectopically expressed RBaK-KΔL, as detected by immunofluorescence staining, was localized to the nucleus (Fig. 7A). The pattern of nuclear staining of RBaK-KΔL was identical to that of full-length RBaK (compare Figs. 4B and 7A). In addition, immunoblotting of lysates of transfected cells detected the high level expression of this mutated form of RBaK-KΔL (Fig. 7B). Therefore, the inability of RBaK-KΔL to decrease BrdUrd incorporation is not due to altered subcellular distribution or low level of protein expression. Instead, it seems to be due either to its lack of an intact Linker motif, which is required to bind to RB, or to its lack of a zinc finger motif, which is presumably required to bind to DNA.

**DISCUSSION**

By using the yeast two-hybrid system, we identified the novel RB-associated KRAB repressor RBaK. This widely expressed protein interacts with RB in yeast and in vitro. Ectopically expressed RB and RBaK have similar effects on DNA synthesis and on the expression of an E2F-dependent promoter in fibroblasts (Fig. 8A). However, a mutant RBaK that cannot bind RB in vitro is unable to prevent entry into S phase in vivo, despite high level expression and nuclear localization. Together, these data suggest a model in which RB represses the expression of genes involved in DNA synthesis by localizing RBaK, a transcriptional repressor, to relevant promoter complexes (Fig. 8B).

**FIG. 8.** Schematic diagram of the proposed RB-RBaK interaction that may repress E2F-dependent genes. A, ectopic expression of RB or RBaK can repress an E2F-dependent promoter and can decrease BrdUrd incorporation in fibroblasts. B, proposed mechanism by which RB localizes RBaK, a transcriptional repressor, to E2F-dependent promoter. There may be other components in this proposed transcriptional repressor complex, which are represented by ? in the diagram. This model is consistent with the data presented here but remains to be formally proved.

The size of the KRAB gene family is remarkable in that it includes well over 100 different members (49, 57). In this family of genes, the KRAB motifs and the zinc fingers are relatively conserved motifs which suggest that they perform common functions. The region that links these motifs, on the other hand, is not very well conserved in different members of this family. As such, it is thought that the amino acid sequence in the linker might define sub-families within the larger KRAB gene family; however, this linker motif has no known function in these proteins. In this regard, it is quite interesting that mouse and human RBaK have similar sequence within the linker motif. This motif has been predicted to form an amphipathic helix that may interact with components of the basal transcriptional factor complex or other cellular proteins (49). Recent studies are beginning to suggest different models for their repressor activity. It is quite well established that the KRAB motif itself harbors the transcriptional repressor activity for these proteins (58–61, 63). This motif has been predicted to form an amphipathic helix that may interact with components of the basal transcriptional factor complex or other cellular proteins (49). Tremendous insight was gained by the finding that KRAB motifs can physically interact with human and mouse RING domain containing proteins known as KAP1/TIF1b and KRIP-1, respectively (73–75). The interaction with
KAP-1 seems to be required for KRAB-mediated repression (73). However, the KRAB motif is necessary and sufficient for the interaction with KAP-1 (73), whereas our studies suggest that the KRAB motif is dispensable for interactions between RBaK and RB (Fig. 3B). Because different motifs of RBaK may mediate interactions with KAP-1 and RB, it is conceivable that KAP-1 may also be a component of a transcriptional repressor complex that is centered on an RB/E2F base as depicted in Fig. 8B.

How KAP-1 functions as a co-repressor for KRAB proteins is not known. However, it was recently shown to bind to homologues of the HP1 gene family of nonhistone heterochromatin-associated proteins (76). Co-localization studies of KAP-1 and certain of these proteins to distinct subnuclear foci suggest that KRAB family members may regulate gene expression in part by a mechanism involving the regulation of chromatin structure (76). As noted above, RB may potentially be linked to the regulation of chromatin structure because it can interact with histone deacetylase HDAC-1 (42, 43). In these studies, RB-associated histone deacetylase activity accounted for a significant portion of RB-mediated gene repression. It is conceivable that the repression of genes by regulating chromatin structure would be an efficient mechanism to maintain gene silencing at particular phases of the cell cycle or during specific developmental stages. Whether KAP-1 or histone deacetylase activity is involved in RBaK-mediated repression of E2F-dependent genes or the cell cycle arrest induced by RB or RBaK will be important questions to address experimentally.

We know of no evidence that other KRAB proteins are directly or indirectly linked to cell cycle control. The finding that RBaK is widely, if not ubiquitously, expressed suggests that its potential role in cell cycle control may be independent of cell type. That RBaK may mediate RB-dependent cell cycle arrest would therefore be consistent with the ability of RB to arrest cell proliferation in a manner that appears to be largely independent of cell type. If RB and RBaK do cooperate to arrest cell proliferation, it would be of interest to determine whether RBaK and RB repress similar sets of genes. The preponderance of evidence indicates that RB functions as a tumor suppressor by physically interacting with members of the E2F family to repress the expression of E2F-dependent genes (reviewed in Refs. 1, 2, and 19). That this is the key tumor suppressor activity of RB is based largely on the correlation between the ability of RB to bind to various E2Fs and inhibit their transactivation function and its ability to prevent cell proliferation. Excellent support of this model is provided by the findings that a mutated form of E2F-1 that disrupts the RB-E2F complex seems to also disrupt the ability of RB to suppress cell proliferation (77). Hence, if RBaK mediates these growth-suppressive effects of RB, E2F-1-dependent promoters would seem to be good candidates for RBaK-mediated repression. It will be important to extend our findings that an artificial reporter can be repressed by RBaK in a manner that depends on E2F-1 DNA-binding sites (Fig. 5) and to address whether RBaK can repress the expression of endogenous genes that are regulated by E2F-1. In addition, it will be important to confirm that this is the mechanism by which RBaK functions to regulate cell proliferation.

There is a precedent for the interactions that we have proposed between RB and a transcriptional repressor. Both RB and the RB-related protein p130 have been shown to bind to a transcriptional repressor, HBP-1 (45). Like RBaK, HBP-1 is a bona fide transcriptional repressor that appears to accomplish RB-related functions, repression of N-myc promoter activation by E2F-1 and inhibition of BrdUrd incorporation. Furthermore, HBP-1 seems to require interaction with RB to repress the N-myc promoter (45). One distinct difference between RBaK and HBP-1 is the absence of a recognized RB-binding motif (such as the LXCXE amino acid motif) in RBaK. This apparent difference suggests that RBaK and HBP-1 may interact with RB through distinct mechanisms. Conceivably, proposed interactions between RB and different transcriptional repressors may provide a mechanism by which RB could contribute to the regulation of diverse cellular processes like cell proliferation and cellular differentiation. Indeed, the forced expression of HBP-1 appears to affect skeletal muscle differentiation (78), a process that is known to involve RB (36, 79). Determining whether the effects of RB on cell proliferation, cell differentiation, and apoptosis are carried out by different potential mediators, such as HDAC-1, HBP-1, and RBaK, will likely lead to new insight into how RB functions as a regulator of these diverse processes.

A model in which RB forms a multimeric complex with a transcriptional repressor and a transcription factor is consistent with a number of studies of RB-mediated repression of E2F-1-dependent genes (24, 44, 80–82). It is also consistent with the observation that RB can repress the activity of other transcription factors as long as it is physically localized to the DNA (81). In the presently accepted model, E2F-1 accomplishes this by localizing RB to relevant promoters (19). Because the potent repressor effects of KRAB proteins may extend over a distance (83), if RBaK were co-localized by E2F-1 and RB, this may actively repress the activity of other transcription factors that may flank E2F-1-binding sites. Such active repression appears to be required for RB-mediated cell cycle arrest because merely inactivating E2F-1-dependent genes does not seem to be sufficient to block cell proliferation (44, 77).

As mentioned above, intact A and B motifs are required for RB to function as an active repressor and to arrest cell proliferation (5, 30). Interestingly, these motifs of RB are required even when the repression is apparently independent of E2F-1, such as the repression of GAL4-dependent gene activation by GAL4-RB (82, 84). With our proposed model, this suggests that the A and B motifs are also required for binding to a putative co-repressor like RBaK. Although this is formally true in the yeast two-hybrid system, it is possible that the A and B motifs are required not merely for RB binding to a repressor like RBaK but also for the formation of a multimeric complex as depicted in Fig. 8B. Previous work has shown that RB can, in fact, form a larger complex with an E2F and another protein, such as a mutated form of adenovirus E1a oncprotein (85). It will be important to establish whether RB, RBaK, and E2F-1 can be found in one complex and to make a correlation between the presence of such a complex and certain mutations in RB and E2F-1 that seem to disrupt the growth-suppressive functions of RB.

It is entirely possible that RB has more than one mechanism for repressing the expression of genes needed for DNA synthesis, in particular cellular contexts. For example, not only is RB important for regulating the transition through G1 into S phase in actively proliferating cells (86), but it is also essential for preserving a G1-arrested state in terminally differentiated cells such as neuronal cells and skeletal myocytes (36, 79, 87, 88). It is conceivable that the mechanism by which RB represses the expression of genes needed for DNA synthesis may differ in proliferating cells and in cells that have terminally ceased proliferating. Whether RBaK has a particular function in one of these specific contexts and the relevance of this for the tumor suppressor function of RB will be addressed in future studies.

One limitation of our studies was our inability to show interaction between RB and Myc epitope-tagged hRBaK in transiently transfected fibroblasts. The absence of this evidence
may reflect either biological mechanisms or limitations of the methods used to detect such an interaction. Perhaps the most likely explanation is that a co-immunoprecipitation assay lacks the sensitivity required to detect what may be a transient interaction in the context of a larger DNA binding complex. More sensitive assays, such as immunoprecipitation-electrophoretic mobility shift assay, which is routinely used to show association between RB and EZF-1 (e.g. Ref. 27), may be required to demonstrate this association in vivo. However, because the consensus DNA binding sequences for RBaK are not known, this approach is not feasible at present. Therefore, as an alternative to showing RB-RBaK association in vivo, we have investigated the functional correlation between the ability of RB to bind RBaK or RBaK-KL in vitro and the relative ability of each to block BrdUrd incorporation (Fig. 3 and Table 1). The correlation we have shown between RB-RBaK complex formation in vitro and the ability of RBaK to decrease BrdUrd incorporation in vitro supports the existence of a RB-RBaK complex that may contribute to RB-mediated repressor activity and cell cycle arrest (Fig. 5B). Further studies will be needed to confirm the existence of such a complex in vivo and to address the importance of RBaK as a mediator of the tumor suppressor function of RB.

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