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Functional Interactions between the hBRM/hBRG1 Transcriptional Activators and the pRB Family of Proteins

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hBRG1 and hBRM are mammalian homologs of the SNF2/SWI2 yeast transcriptional activator. These proteins exist in a large multisubunit complex that likely serves to remodel chromatin and, in so doing, facilitates the function of specific transcription factors. The retinoblastoma protein (pRB) inhibits cell cycle progression by repressing transcription of specific growth-related genes. Using the yeast two-hybrid system, we demonstrate that the members of the hBRG1/hBRM family of proteins interact with the pRB family of proteins, which includes pRB, p107, and p130. Interaction between the hBRG1/hBRM family with the pRB family likely influences cellular proliferation, as both hBRG1 and hBRM, but not mutants of these proteins unable to bind to pRB family members, inhibit the formation of drug-resistant colonies when transfected into the SW13 human adenocarcinoma cell line, which lacks endogenous hBRG1 or hBRM. Further, hBRM and two isoforms of hBRG1 induce the formation of flat, growth-arrested cells in a pRB family-dependent manner when introduced into SW13 cells. This flat-cell-inducing activity is severely reduced by cotransfection of the wild-type E1A protein and variably reduced by the cotransfection of mutants of E1A that lack the ability to bind to some or all members of the pRB family.

The retinoblastoma protein (pRB) functions within a complex network of proteins needed for the tight regulation of the mammalian cell cycle. In addition to pRB and its family members, this network includes the cyclin-dependent kinases and the inhibitors of these kinases (reviewed in reference 53). pRB works to negatively influence the cell's progression through G_1 into the S phase of the cell cycle by binding to and regulating the activities of many transcription factors important for growth control, such as E2F (1, 9, 30, 31, 35). Only underphosphorylated pRB, found predominantly in G_1, can bind to its cellular partners. As the cell cycle progresses, the cyclin-dependent kinases interact with and are activated by specific cyclin regulatory subunits (reviewed in reference 46) to induce increased phosphorylation of pRB on serine and threonine residues and the subsequent elimination of its ability to bind to its cellular partners (5, 12, 18, 24, 29, 43, 45). Thus, pRB represents an important intermediary between the cell cycle proteins and the transcriptional machinery that regulates growth progression.

pRB requires a domain termed the A/B pocket in order to interact with many of its cellular binding proteins and exert its growth suppression. Indeed, most retinoblastoma tumors involve mutations in the A/B pocket that disrupt pRB's ability to bind to proteins such as E2F (33). Further, viral oncoproteins such as simian virus 40 large T antigen, adenovirus E1A, and human papillomavirus E7 may transform cells in part by binding to the A/B pocket, rendering pRB incapable of binding to and negatively regulating cellular factors, like E2F, that are important for growth progression (10, 30, 35). Thus, either the sequestration of pRB by viral oncoproteins or mutations in the A/B pocket can eliminate normal pRB function and contribute to oncogenesis.

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pRB and two of its homologs, p107 and p130, comprise the pRB family of proteins. Both p107 (57) and p130 (28, 41, 44) also contain an A/B pocket that allows binding to the viral oncoproteins. As well, p107 and p130 share a spacer sequence between the A and B subdomains of the pocket that mediates their interaction with cyclins A and E (23, 25). All pRB family members complex with various affinities for different E2F family members, and each pRB family member/E2F complex likely has a specific time during the cell cycle during which it is functionally relevant (7, 15, 16). Perhaps because both p107 and p130 complex with E2F family members, they, like pRB, have growth-suppressive properties in cell culture (14, 57). While p107 has yet to be found altered in any malignancies, p130 has been mapped to a chromosomal region (16q13) that undergoes allelic loss in a variety of human tumors (28, 41).

pRB likely functions through its numerous interactions with different cellular proteins such as MyoD (26), c-Abl (54), and MDM2 (56), in addition to the E2F family members. We previously reported that pRB interacts both in vitro and in vivo with the human brahma-related gene 1 protein (hBRG1) (19). hBRG1 and its family member hBRM are mammalian homologs of the yeast SNF2/SWI2 transcriptional activator and *Drosophila* brahma, all sharing a domain identified in a large group of DNA helicases and ATPases (36, 40, 47, 52) and a bromodomain that is also found in many nuclear proteins such as the E1A-binding protein p300 (21). In the yeast *Saccharomyces cerevisiae*, SNF2/SWI2 is required for the activation of many genes and displays an DNA-dependent ATPase activity (38, 39). The SN2/SWI2 family members do not bind to specific sequences in promoters but instead act within a large multiprotein complex (the SWI/SNF complex) to assist the DNA binding of specific transcription factors, likely by altering the structure of chromatin (8, 49). The yeast SWI/SNF complex is composed of at least 10 subunits (including SNF2/SWI2) and significantly stimulates the binding of GAL4 derivative proteins to nucleosomal DNA (6, 17). hBRG1 can be partially purified as part of a 2×10^6-Da complex (the human SWI/SNF

complex) that contains at least eight other proteins (36). The human SWI/SNF complex containing hBRG1 and hBRM has been shown to mediate an ATP-dependent disruption of nucleosomes, allowing the binding of specific factors to nucleosomal DNA (34, 37).

hBRG1 interacts with hypophosphorylated pRB only, and this interaction is dependent on an intact A/B pocket. hBRG1 bears an LXCXE motif found in many pRB-interacting proteins, such as human papillomavirus E7, simian virus 40 large T antigen, and adenovirus E1A. This motif, the E7 homology region, is necessary for the interaction between the viral oncoproteins and the pRB family (3, 20, 22, 48, 55). Deletion of this E7 homology region from hBRG1 completely eliminates its ability to bind to pRB. Interestingly, the transfection of hBRG1 into the human carcinoma cell line SW13, which contains no hBRG1 (or hBRM), induces the formation of flattened, nondividing cells (19). This flat-cell induction is dependent on the hBRG1-pRB interaction because an hBRG1 mutant that cannot bind pRB (hBRG1ΔE7) cannot induce flat cells, and the cotransfection of E1A with hBRG1 eliminates flat-cell formation, presumably by sequestering pRB. hBRG1 likely induces SW13 flat cells by cooperating with other members of the pRB family, as the cotransfection of hBRG1 with a pRB-nonbinding mutant of E1A (E1A;928) partially reduces (but does not eliminate) SW13 flat-cell formation (19). This reduction in flat-cell formation may result from E1A;928's ability to sequester either p107 or p130 or both. Others have shown that pRB interacts with hBRM in order to potentiate glucocorticoid receptor-mediated transcriptional activation (51). This effect is believed to involve a direct interaction between hBRM and pRB, since it requires an intact A/B pocket in pRB and is sensitive to a mutation within the LXCXE motif of hBRM.

In this report, using the yeast two-hybrid system, we demonstrate that both hBRG1 and hBRM are capable of binding to pRB and other members of the pRB family. Further, hBRM, like hBRG1, possesses SW13 flat-cell-inducing activity that is pRB family dependent. Also, two isoforms of hBRG1 that result from alternative splicing show different binding affinities for the pRB family and correlative SW13 flat-cell activity. Finally, using other mutants of E1A that differ in the ability to bind pRB family members, we examine the relative effects of pRB family sequestration on the flat-cell activities of both hBRG1 and hBRM.

MATERIALS AND METHODS

Yeast two-hybrid system. hBRG1a was constructed by overlap extension PCR to remove the alternative exon. Two separate reactions were performed; one used PCR primer pair GTCTTCCTCCTCATCCTGCTCCTCGTC and CGCGGATCCTGTACCGTGAACAGCGTGGAGG, and the second used the primer pair CAGGATGAGGAGGAAGACGAGGTGCCC and GTCCTTGTACTTGATCACGG. The products of these two separate reactions were then mixed, and a third PCR amplification was performed with solely the two outside primers of the first two reactions. The final product was sequenced and then subcloned into the full-length hBRG1 cDNA. Homologous portions of hBRG1a, hBRG1b, and hBRM were amplified by PCR and subcloned into plasmid pGADNOT (42), which encodes the GAL4 transcriptional activation domain. PCR primers for the construction of pGAD-hBRM-CT were GCGGCCGCAACGTGGATCAGAAAGTGATCCAGGC and GACGCGTCGACCTCATCATCCGTCCCACTTCC, using hBRM as the template. PCR primers for the construction of pGAD-hBRG1a-CT or pGAD-hBRG1b-CT were GCGGGATCCTCAACGTGGACCAGAAGGTGATCCAGGC and GACGCGTCGACGTCTTCTTCGCTGCCACTTCCTG, using either hBRG1a or hBRG1b as the template (hBRG1a and hBRG1b differ in the presence and absence, respectively, of a 33-amino acid [aa] sequence C terminal to aa 1255 in hBRG1). All pGAD-hBRG1-CT and pGAD-hBRM-CT constructs and their derivatives involve the fusion of the GAL4 activation domain to C-terminal protein sequences beginning with amino acid sequence NVDQKVI. . . found at aa 1220 in hBRG1 and aa 1189 in hBRM and include all sequences to the C-terminal end of each protein (including the bromodomain). pGAD-hBRG1bΔE7-CT incorporates an in-frame deletion of aa 1301 to 1411 from hBRG1a. pGAD-hBRMΔE7-CT incorporates an in-frame deletion of aa 1265 to 1338 from hBRM. pSH2-RB contains the full-length *Rb* cDNA subcloned into pSH2-1 plasmid containing the LexA DNA-binding domain (19, 27). Both pSH2-107 and pSH2-130 were constructed by PCR amplification of portions of pCMV-107 and pCMV-130 and include the A/B pocket and C-terminal domains of p107 and p130 subcloned into pSH2-1. PCR primers for the construction of pSH2-107 were GCGGGATCCTCGAAGCAGTCATTACTCCTGTTGC and GACGCGTCGACTGCTCTTTCACTGACAACATCCTG. PCR primers for the construction of pSH2-130 were GCGGGATCCTCAGCCCTTGTGTGACTCCAGTTTC and GACGCGTCGACTCAGTGGGAACCACGGTCATTAGC. pSH2-107 includes the LexA DNA-binding domain fused to all sequence C terminal to aa 247 of p107 (beginning at EAVITPV. . .). pSH2-130 includes the LexA DNA-binding domain fused to all sequence C-terminal to aa 355 of p130 (beginning at SPCVTPV. . .). pSH2-107 F846, pSH2-107 DE, and pSH2-107 EC incorporate mutations previously described (57). Two-hybrid assays for protein interactions were performed in *S. cerevisiae* CTY10-5d, and 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal) assays were performed as described previously (42).

Glutathione *S*-transferase (GST) in vitro binding assays. pGEX-BRG1-E7 was constructed by subcloning PCR-amplified cDNA encoding aa 1202 (beginning at CTVNSVEE. . .) through aa 1335 of hBRG1a into the pGEX bacterial expression plasmid (Pharmacia). pGST-BRM-E7 was constructed by subcloning PCR-amplified cDNA encoding aa 1171 (beginning at CTVNSVEE. . .) through aa 1305 of hBRM into pGEX. The upstream PCR primer for both pGST-BRG1-E7 and pGST-BRM-E7 was CGCGGATCCTGTACCGTGAACAGCGTGGAGG. The downstream PCR primer for pGEX-BRG1-E7 was CCGGAATTCGGAGCAGCCGAACATCTTCTCCTC. The downstream PCR primer for pGST-BRM-E7 was CCGGAATTCGGACCCCCTCCCAAATATTTTCTC. pGST-BRG1a and pGST-BRG1b were constructed by subcloning PCR-amplified cDNA of either hBRG1a or hBRG1b encoding aa 1202 through 1411 of hBRG1 into pGEX. PCR primers for pGST-BRG1a and pGST-BRG1b were CGCGGATCCTGTACCGTGAACAGCGTGGAGG and GTCCTTGTACTTGATCACGG. pGST-BRG1a-CT, pGST-BRG1b-CT, and pGST-BRM-CT were constructed by subcloning the cDNA fragments of pGAD-hBRG1a-CT, pGAD-hBRG1b-CT, and pGAD-hBRM-CT into the pGEX-3XPL bacterial expression vector. Binding assays were performed as described previously (19).

Transfections and protein extraction. Transfection of cells with all mammalian expression constructs was performed by the calcium phosphate precipitation method (11). Proteins for Western blot (immunoblot) analysis were harvested 48 h after transfection. Extraction of hBRG1 and its derivatives was performed as described previously (19).

Cell culture. SW13 cells and C33A cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin-streptomycin, and L-glutamine. EC109 cells were cultured in RPMI supplemented with 10% fetal calf serum, penicillin-streptomycin, and L-glutamine.

Flat-cell assays. Confluent plates of SW13 cells were passaged 24 h prior to transfection. Transfection was performed with cells at 40 to 70% confluency. Three micrograms of plasmids encoding hBRG1 or hBRM (or their derivatives) was cotransfected with 2 μg of pBabe-puro. The total amount of DNA transfected was always 20 μg, and the difference was made up with sonicated salmon sperm DNA. Cells were washed 12 to 16 h after precipitation of DNA, cultured for another 24 h without selection, and then put under puromycin (2 μg/ml) selection for 5 days. Flat cells were counted under ×40 magnification, and counting was done blind with respect to transfected DNAs.

Colony formation assays. Assays for the suppression of SW13 colony formation were performed identically to SW13 flat-cell assays except that cells were cultured in puromycin for 7 days. Colonies containing a minimum of eight cells were scored under ×40 magnification.

RESULTS

hBRM binds directly to pRB in vitro and in the yeast two-hybrid system. Previously, we demonstrated that a small sequence encompassing 135 aa of the murine mBRG1 protein (mBRG1c) was able to bind pRB in vitro (19). This sequence contains significant homology to the motif of the human papillomavirus E7 protein known to be critical for the direct interaction between E7 and pRB. Both this motif, $EX_{2-4}LXCXE$, and the sequence adjacent to it are almost entirely conserved between hBRG1 and hBRM. Deletion of the E7 homology motif and the sequence flanking it eliminated hBRG1's ability to bind pRB (19). To show that this binding also can occur between hBRM and pRB, we constructed a GST fusion protein containing 134 aa of hBRM containing its E7 homology motif, designated GST-BRM-E7. This protein was then immobilized on glutathione-Sepharose beads, exposed to lysates of C33A cells transfected with wild-type pCMV-RB, and washed with

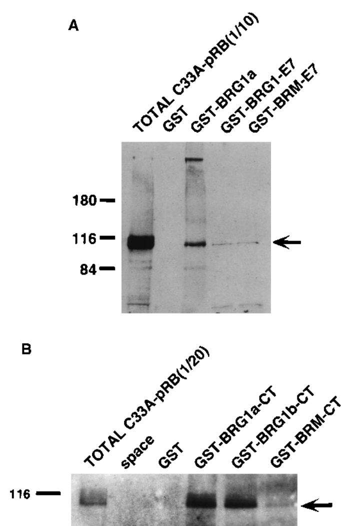

FIG. 1. (A) pRB binds to homologous regions of hBRG1 and hBRM. GST fusion proteins were exposed to extracts of C33A cells transfected with pCMV-RB. Bound protein was washed in binding buffer, boiled in protein gel loading buffer, separated by SDS-PAGE (7.5% gel), and transferred to nitrocellulose. The immunoblot was developed with anti-RB antibody PMG3-245. The arrow indicates pRB migration. The leftmost lane contains 10% of the extract exposed to GST-fusion proteins. (B) pRB binds to larger fragments of hBRG1 and hBRM that include both the E7 homology domain and the bromodomain. The assay was performed essentially as described for panel A except that the total extract lane contains 5% of extract exposed to GST-fusion proteins. The arrow indicates pRB migration. Sizes are indicated in kilodaltons.

lysis buffer. Bound protein was then eluted in sodium dodecyl sulfate (SDS) protein loading buffer, boiled, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then analyzed by immunoblotting with anti-pRB antibody PMG3-245. Figure 1A demonstrates that both GST-BRM-E7 and its hBRG1 homolog, GST-BRG1-E7, specifically bound pRB in vitro, but not as well as a larger (210-aa) fragment of hBRG1a (GST-BRG1a) that contains more sequence flanking the E7 homology motif.

To demonstrate that a larger fragment of either hBRG1 or hBRM could bind pRB in vitro, a region including both the E7 homology domain and the adjacent C terminus of hBRG1/hBRM (including the bromodomain) was fused to GST and expressed in bacteria. Two different isoforms of hBRG1 (GST-BRG1a-CT and GST-BRG1b-CT; described below) and the homologous fragment of hBRM (GST-BRM-CT) all bound to pRB from lysates of C33A cells transfected with pCMV-RB (Fig. 1B). Interestingly, GST-BRM-CT bound pRB significantly less well than either isoform of GST-BRG1-CT. All three GST fusion proteins were equally stable when expressed in bacteria and subsequently isolated using glutathione-Sepharose (data not shown).

To reinforce these binding data, we used the yeast two-hybrid system by constructing a plasmid expressing hBRM fused to the GAL4 activation domain (pGAD-hBRM-CT). This plasmid contains sequences 3′ to the ATPase domain of hBRM (identical to the fragment incorporated in GST-BRM-CT, described above), including the E7 homology region and the bromodomain. Cotransformation of *S. cerevisiae* CTY10-5d with pGAD-hBRM-CT and a plasmid expressing full-length pRB fused to the LexA DNA-binding domain (pSH2-RB) resulted in activation of the *lacZ* reporter gene, indicating that the two fusion proteins interact (Table 1). Control experiments demonstrated that pGAD-hBRM-CT could not interact with the LexA DNA-binding domain alone and that pSH2-RB could not interact with the GAL4 activation domain alone. As expected, the deletion of the E7 homology region of hBRM eliminated its ability to interact with pRB in *S. cerevisiae.*

hBRG1 and hBRM bind to other pRB family members in the yeast two-hybrid system. hBRG1 function most likely depends on interactions with not only pRB but also other pRB family proteins. When cotransfected with hBRG1, the pRB-nonbinding E1A;928 mutant blocked a portion of the flat-cell activity, implying that this mutant in part might inhibit flat-cell formation by binding the other pRB family proteins (19). To test this possibility directly, we constructed plasmids expressing portions of both p107 and p130 fused to the LexA DNA-binding domain (pSH2-107 and pSH2-130). These constructs include all sequences encoding the E1A-binding pocket and C terminus of p107 and p130 (Fig. 2). These plasmids, along with pSH2-RB, were cotransfected into *S. cerevisiae* with pGAD-hBRM-CT and pGAD-hBRG1a-CT. pGAD-hBRG1a-CT is the homolog of pGAD-hBRM-CT and also represents one of the two identified alternatively spliced forms of hBRG1 (described below). Table 1 shows that in the yeast two-hybrid system, both pGAD-hBRG1a-CT and pGAD-hBRM-CT could interact with p107 as strongly as they do with pRB. As well, mutations that disrupt the pocket region of p107 completely eliminated its ability to interact with either pGAD-hBRG1a-CT or pGAD-hBRM-CT. Interestingly, pGAD-hBRG1a-CT bound to p130 less well than it did to p107 and pRB, and pGAD-hBRM-CT showed negligible binding to p130. Further, in this system, pGAD-hBRG1a-CT always bound with a greater affinity to pRB and its family members than did pGAD-hBRM-CT. As expected, all positive interactions were eliminated if the E7 homology domain is removed from either pGAD-hBRG1a-CT or pGAD-hBRM-CT, and neither the LexA

TABLE 1. Yeast two-hybrid analysis of hBRG1/hBRM binding to the pRB family[a]

Plasmid	Staining						
	pSH2-1	pSH2-RB	pSH2-107	pSH2-130	pSH2-107 DE	pSH2-107 EC	pSH2-107 F846
pGAD-hBRG1a-CT	−	+++	+++	+	−	−	−
pGAD-hBRG1b-CT	−	+	+	−	−	−	−
pGAD-hBRG1bΔE7-CT	−	−	−	−	−	−	−
pGAD-hBRM-CT	−	++	++	−	−	−	−
pGAD-hBRMΔE7-CT	−	−	−	−	−	−	−
pGADNOT	−	−	−	−	−	−	−

[a] *S. cerevisiae* CTY10-5d was cotransformed with two plasmids, one expressing a GAL4 activation domain fusion protein and the other expressing a LexA DNA-binding fusion protein. Transformants were selected on SC-His-Leu plates and subjected to X-Gal blue/white assay. +++, blue staining after approximately 2 h; ++, staining after approximately 4 h; +, staining after approximately 6 h; −, no staining. pSH2-1 is the parent vector for the LexA fusion constructs. pGADNOT is the parent vector for the GAL4 fusion constructs.

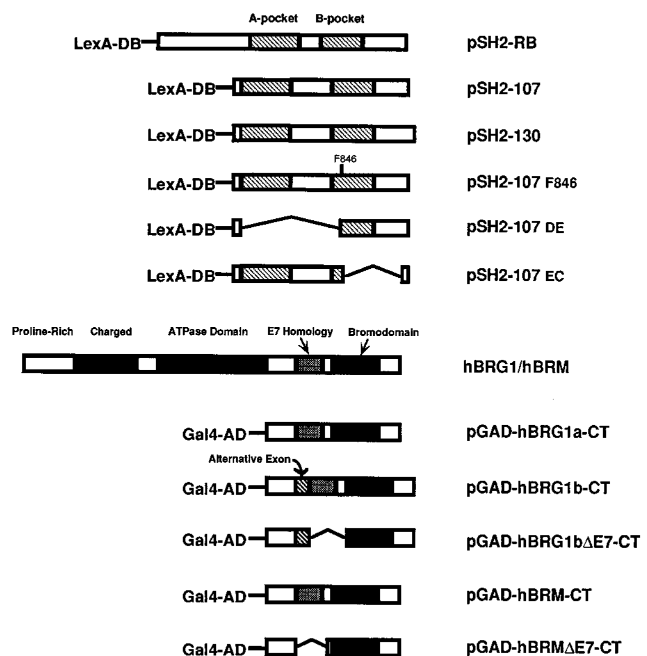

FIG. 2. Constructs used for yeast two-hybrid analysis. A diagram of full-length hBRG1/hBRM is included for reference. LexA-DB, LexA DNA-binding domain.

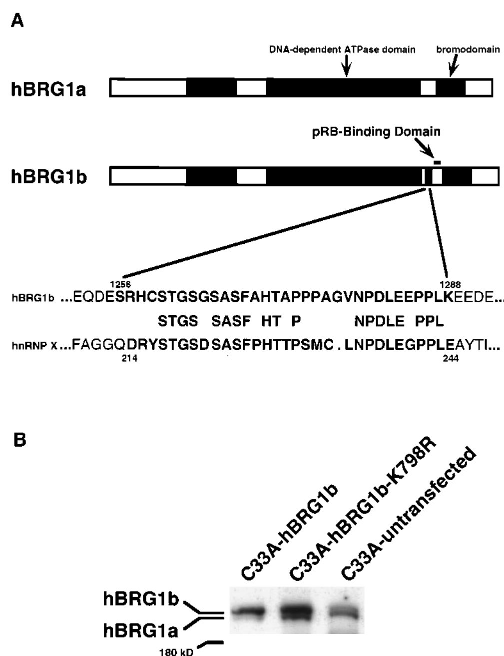

FIG. 3. Comparison of hBRG1a and hBRG1b. (A) *hBRG1b* contains an additional 99 bp exon just 5′ to the E7 homology domain. The translated 33-aa product of this exon in *hBRG1b* is depicted in boldface, with surrounding sequence in plain text. The translated product of the alternatively spliced 93-bp exon in the gene for the hnRNP X protein is aligned with hBRG1b. (B) Extracts from untransfected C33A cells and cells transfected with either wild-type hBRG1b or hBRG1b K798R demonstrate the presence of proteins with two different electrophoretic mobilities when separated by SDS-PAGE (7.5% gel). The immunoblot was developed with anti-hBRG1/hBRM antibody J1.

DNA-binding domain nor the GAL4 activation domain could alone interact with another protein analyzed here.

Different pRB family binding and functional activity of two hBRG1 isoforms. hBRG1 is expressed as two different messages, hBRG1a and hBRG1b. hBRG1b includes an alternatively spliced 99-bp exon just upstream of the E7 homology region, while hBRG1a does not (13) (Fig. 3A). These mRNAs should encode proteins of slightly different sizes (approximately 4-kDa difference). Indeed, immunoblot analysis shows that there are two detectable hBRG1 proteins in C33A cells differing slightly in electrophoretic mobility (Fig. 3B). C33A cells have been shown to contain no endogenous hBRM (47), and therefore it is likely that the J1 anti-hBRG1/hBRM antibody used for this analysis detects only hBRG1 proteins.

Because the additional 33-aa sequence in hBRG1b lies immediately N terminal to the defined pRB family binding domain, we examined the relative abilities of the two hBRG1 proteins to bind the pRB family. A cDNA containing the C terminus of hBRG1b including the alternative exon was subcloned into the pGADNOT yeast expression vector. Except for the presence of the alternative exon, this construct, pGAD-hBRG1b-CT, is identical to pGAD-hBRG1a-CT. As shown in Table 1, in the two-hybrid system, pGAD-hBRG1b-CT also bound to pRB and p107, but with a significantly weaker affinity. Interestingly, unlike pGAD-hBRG1a-CT, pGAD-hBRG1b-CT did not detectably bind to p130 in the yeast two-hybrid system.

To test whether these results also would be obtained in vitro, we constructed GST fusion proteins of both spliced forms of hBRG1 and analyzed their abilities to bind pRB from lysates of the RB^+ esophageal carcinoma cell line EC109. Figure 4 shows that a GST fusion protein containing both the hBRG1 E7 homology region and the spliced exon (GST-BRG1b) bound to pRB less well than GST-BRG1a, which lacks the exon. This binding difference was also demonstrated by using *RB* expressed in a different cell line and using the larger fragments hBRG1a-CT and hBRG1b-CT that include both the E7 homology region and the bromodomain. GST-BRG1a-CT bound to pRB from lysates of pCMV-RB-transfected C33A cells better than GST-BRG1b-CT (Fig. 1B).

The human adrenal carcinoma cell line SW13, while apparently RB^+, does not detectably express either hBRG1 or hBRM (19, 47). As previously described, the transfection of hBRG1-expressing plasmids into SW13 cells induced the formation of flattened, growth-arrested cells. hBRG1 requires an interaction with either pRB or its family members in order to induce flat SW13 cells, and the presence of the alternative exon reduced the binding between hBRG1b and the pRB family. Therefore, we tested the possibility that hBRG1b has reduced flat-cell-inducing activity in SW13 cells. Table 2 dem-

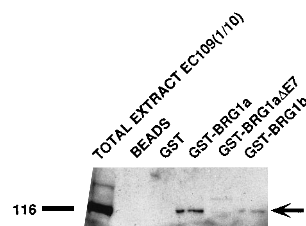

FIG. 4. Comparison of in vitro binding of GST-BRG1a to GST-BRG1b to pRB. GST fusion proteins were exposed to extracts of the esophageal carcinoma cell line EC109. Bound protein was washed, boiled in protein gel loading buffer, separated by SDS-PAGE (7.5% gel), and transferred to nitrocellulose. The immunoblot was developed with anti-RB antibody PMG3-245. The arrow indicates pRB migration. The leftmost lane contains 10% of the extract exposed to GST fusion proteins. Size is indicated in kilodaltons.

TABLE 2. SW13 flat-cell assay: hBRG1a versus hBRG1b

Plasmid transfected[a]	No. (%) of flat cells[b]				
	Expt 1	Expt 2	Expt 3	Expt 4	Avg % flat cells
hBRG1a	99 (100)	137 (100)	75 (100)	56 (100)	100
hBRG1b	71 (72)	94 (69)	31 (41)	35 (63)	61
pBJ5	9 (9)	7 (5)	0 (0)	1 (2)	4

[a] SW13 cells were transfected with either isoform of hBRG1 or with pBJ5, the parent mammalian expression vector. Three micrograms of each plasmid was introduced to cells with 2 μg of pBabe-puro, and the difference was brought up to 20 μg by using 10 μg of sonicated salmon sperm DNA and 5 μg of pSVE empty expression vector.
[b] Total number of flat cells in 20 random 40× fields. Counting was done blind with respect to the transfected plasmid.

TABLE 4. SW13 flat-cell assay: cotransfection of E1A with hBRM

Plasmids transfected[a]	No. (%) of flat cells			
	Expt 1	Expt 2	Expt 3	Avg % flat cells
hBRM + SVE	81 (100)	33 (100)	42 (100%)	100
hBRM + SV-E1A	0 (0)	1 (3)	0 (0%)	1
hBRM + SV-E1A;928	32 (40)	8 (24)	9 (21%)	28
pCG + SVE	6 (7)	2 (6)	0 (0%)	4
pCG + SV-E1A	ND[b]	0 (0)	ND	0
pCG + SV-E1A;928	ND	1 (3)	ND	3

[a] SW13 cells were transfected with 3 μg of hBRM and 5 μg of either SV-E1A or SV-E1A;928. pCG is the empty mammalian expression vector for hBRM. SVE is the empty expression vector for E1A and E1A;928. All other methods were as described for Table 2.
[b] ND, not determined.

onstrates that hBRG1b repeatably induced fewer flat cells than hBRG1a. This result, while not dramatic, correlates well with our pRB binding data and further supports our other data indicating the importance of the ability of hBRG1 to bind to pRB (or the pRB family) in order to arrest SW13 cells.

hBRM demonstrates pRB-dependent flat-cell activity in SW13 cells. hBRM is highly homologous to hBRG1 and likely functions similarly. Therefore, we tested hBRM's ability to induce the formation of SW13 flat cells. Table 3 shows that transfected hBRM, like hBRG1, had SW13 flat-cell-inducing activity. This activity likely is dependent on pRB, as a pRB-nonbinding mutant of hBRM (hBRMΔE7) lacked flat cell activity. Many other aspects of hBRM function must be required for flat cell induction, as other mutants of hBRM (described in reference 47), hBRM K798R, an ATP-binding mutant, hBRMΔ3, lacking a large portion of sequence N-terminal to the ATP-binding motif, and hBRMΔ4, lacking sequence C-terminal to the E7 homology domain, had negligible flat-cell-inducing activity.

The cotransfection of hBRM with the adenovirus E1A protein, which sequesters pRB (and its family members p107 and p130), eliminated flat-cell formation (Table 4). In addition, the cotransfection of hBRM with a mutant E1A protein (E1A;928) that is unable to bind to pRB only partially inhibited flat-cell formation. Therefore, hBRM, like hBRG1, most likely depends on interactions with other pRB family proteins (that are still bound by E1A;928) in order to have full flat-cell-inducing activity.

hBRG1 and hBRM reduce the formation of drug-resistant SW13 colonies. During transformation of cell lines with a mixture of expression constructs and a drug resistance gene, cells receiving only the drug resistance marker can form viable colonies with normal morphology. When introduced into cells together with drug resistance genes, both p53 and pRB can reduce the formation of drug-resistant colonies of recipient cell lines that lack functional alleles of these respective tumor suppressors (2, 50). Similarly, hBRG1a and hBRM, when introduced into cells with the puromycin resistance gene, could significantly suppress the colony formation of SW13 cells cultured in puromycin for 7 days (Table 5). Further, this suppression was pRB dependent, as both hBRG1aΔE7 and hBRMΔE7 were severely defective in the ability to inhibit the formation of drug-resistant SW13 colonies. This assay further corroborates the SW13 flat-cell assay and implies a direct relationship between the hBRG1/hBRM family's ability to bind to the pRB family and its ability to inhibit cellular proliferation.

Effects of E1A and E1A mutants on hBRG1 and hBRM SW13 flat-cell activity. Previously, we demonstrated that E1A; 928 reduced but did not eliminate the SW13 flat-cell activity of either cotransfected hBRG1 or hBRM. A number of other E1A mutants have variable pRB family binding profiles (3, 4). In experiments similar to our initial flat-cell assay, we cotransfected constructs that express E1A (from human adenovirus type 5) and four mutant E1A proteins with various pRB family member binding profiles in order examine which interactions between hBRG1 or hBRM and the pRB family are important for flat-cell induction. Table 6 demonstrates that when cotransfected into SW13 cells with either hBRG1 or hBRM, wild-type E1A was able to reduce flat-cell formation to approximately one-fourth to one-third of that induced by hBRG1a, hBRG1b, or hBRM expressed with empty vector. Wild-type E1A did not completely suppress flat-cell formation in these experiments because the E1A gene was expressed from the native adenovirus promoter, not the stronger simian virus 40 pro-

TABLE 3. SW13 flat-cell assay: hBRM

Plasmid transfected[a]	No. (%) of flat cells[b]			
	Expt 1	Expt 2	Expt 3	Avg % flat cells
hBRM	72 (100)	36 (100)	60 (100)	100
hBRM-K798R	4 (6)	2 (6)	0 (0)	4
hBRMΔE7	6 (8)	3 (8)	9 (15)	10
hBRMΔ3	0 (0)	1 (3)	0 (0)	1
hBRMΔ4	4 (6)	2 (6)	15 (25)	12
pCG	4 (6)	1 (3)	0 (0)	3

[a] SW13 cells were transfected with hBRM, mutants of hBRM, or pCG, the parent mammalian expression vector. Three micrograms of each plasmid was introduced to cells with 2 μg of pBabe-puro, and the difference was brought up to 20 μg by using 15 μg of sonicated salmon sperm DNA.
[b] Counting was done as for Table 2.

TABLE 5. SW13 colony formation assay

Plasmid transfected[a]	No. (%) of colonies[b]			
	Expt 1	Expt 2	Expt 3	Avg % colonies
pBJ5	52 (100)	49 (100)	98 (100)	100
hBRG1a	6 (12)	3 (6)	4 (4)	7
hBRG1aΔE7	35 (67)	38 (78)	47 (48)	65
pCG	39 (100)	32 (100)	63 (100)	100
hBRM	12 (31)	9 (28)	13 (21)	27
hBRMΔE7	41 (105)	59 (184)	82 (130)	140

[a] SW13 cells were transfected with 3 μg of either hBRG1 or hBRM, 2 μg of pBabe-puro, and salmon sperm DNA.
[b] Total number of colonies in 20 random 40× fields. Counting was done blind with respect to the transfected plasmid.

TABLE 6. SW13 flat-cell assay: cotransfection of E1A and E1A mutants

Assay	Cotransfection[a]	No. (%) of flat cells			
		Expt 1	Expt 2	Expt 3	Avg % flat cells
A	hBRG1a with:				
	Empty vector	95 (100)	55 (100)	85 (100)	100
	Wild-type E1A	19 (20)	23 (42)	21 (25)	29
	E1A *dl*1104	68 (72)	49 (89)	58 (68)	76
	E1A *dl*1107	76 (80)	71 (129)	77 (90)	100
	E1A *dl*1108	32 (34)	13 (24)	22 (26)	28
	E1A *dl*1109	38 (40)	39 (71)	58 (68)	60
B	hBRG1b with:				
	Empty vector	90 (100)	117 (100)	116 (100)	100
	Wild-type E1A	11 (12)	60 (51)	ND[b]	32
	E1A *dl*1104	89 (99)	28 (24)	50 (43)	55
	E1A *dl*1107	58 (64)	93 (79)	45 (39)	61
	E1A *dl*1108	32 (36)	29 (25)	41 (35)	32
	E1A *dl*1109	21 (23)	72 (62)	59 (51)	45
C	hBRM with:				
	Empty vector	133 (100)	73 (100)	133 (100)	100
	Wild-type E1A	24 (18)	31 (42)	44 (33)	31
	E1A *dl*1104	32 (24)	38 (52)	59 (44)	40
	E1A *dl*1107	26 (20)	15 (21)	23 (17)	19
	E1A *dl*1108	20 (15)	26 (36)	49 (37)	29
	E1A *dl*1109	27 (20)	ND	42 (32)	26

[a] SW13 cells were cotransfected with 3 μg of either hBRG1a (assay A), hBRG1b (assay B), or hBRM (assay C), 10 μg of E1A or the indicated E1A mutant, and sonicated salmon sperm DNA. Binding profiles of E1A and its mutants: E1A binds pRB, p107, p130, and p300; E1A *dl*1104 binds p107; E1A *dl*1107 binds p107 and p300; E1A *dl*1108 binds only p300; E1A *dl*1109 binds p107 and p300.

[b] ND, not determined.

moter used in our previous experiments. When cotransfected with hBRG1a, all E1A mutants showed some ability to reduce flat-cell formation relative to empty vector (Table 6, assay A). Two of the E1A mutants, *dl*1104 and *dl*1107, only slightly reduced flat-cell formation (compared with wild-type E1A). Two others, *dl*1108 and *dl*1109, showed a more extreme reduction of hBRG1a's flat-cell activity, with E1A *dl*1108, a mutant that binds only p300, reducing this activity as well as wild-type E1A. The alternatively spliced variant hBRG1b had a similar yet greater sensitivity to mutant E1A cotransfection (Table 6, assay B). Interestingly, all mutants of E1A when cotransfected with hBRM virtually eliminated flat-cell formation (relative to cotransfected wild-type E1A) (Table 6, assay C). The effects seen here were not due to variations in protein levels, as hBRG1a, hBRG1b, and hBRM, as judged by immunoblot analysis with the anti-hBRG1/hBRM antibody J1, were stably expressed at similar levels in SW13 cells when cotransfected with the various E1A proteins (data not shown).

DISCUSSION

In this study, we have built on our previous finding that hBRG1 functionally complexes with pRB in order to induce flat, growth-arrested SW13 cells. In the yeast two-hybrid system, hBRG1 also could interact with the other pRB family members p107 and p130. *hBRG1* is expressed as two alternatively spliced transcripts, and the translated products of these two messages differ in the ability to both interact with the pRB family and induce SW13 flat cells. Specifically, hBRG1a, lacking an additional 33-aa sequence just N terminal to the E7 homology domain, binds to all three pRB family members quite strongly in the yeast two-hybrid system. hBRG1b, on the other hand, shows a significantly weaker ability to bind both to pRB and to p107 and does not bind detectably to p130. Such a difference in binding likely accounts for hBRG1a's twofold-greater flat-cell-inducing activity than that of hBRG1b.

By analyzing other identified sequences in GenBank, we discovered one other protein, heterogeneous nuclear ribonucleoprotein particle (hnRNP) X, that shares significant homology (60% amino acid identity) with the alternative exon found in hBRG1b (Fig. 3A). Like the spliced exon of hBRG1b, this sequence is alternatively spliced into hnRNP X in the form of a 93-bp exon encoding 31 aa (32). hnRNP X shows no other discernible homology with hBRG1b and did not bind to pRB in vitro (data not shown). While altering the adjacent hBRG1b E7 homology domain's ability to interact with the pRB family, this conserved domain may mediate an unknown additional function common to both hBRG1b and hnRNP X.

hBRM also interacted with pRB and p107 in the two-hybrid system, but at a lower level than its homolog hBRG1a. hBRM did not detectably bind to p130. hBRM also demonstrated SW13 flat-cell activity, and this activity was eliminated by both the deletion of the hBRM E7 homology region (hBRMΔE7) and the cotransfection of E1A. Further, the cotransfection of hBRM with the pRB-nonbinding mutant E1A;928 only partially restored flat-cell activity, indicating that hBRM must interact with other pRB family members (likely p107) in order to induce flat cells.

The yeast two-hybrid binding data, while qualitative, correlate well with the SW13 flat-cell data. Both hBRG1a and hBRM interacted significantly with pRB and p107 and similarly demonstrated strong flat-cell activity. hBRG1b bound less well to pRB and p107, and not to p130, and exhibited a flat-cell activity lower than that of hBRG1a. Finally, both hBRG1bΔE7 (19) and hBRMΔE7 could not interact with the pRB family and could not induce flat cells. These data indicate the importance of the hBRG1/hBRM family's ability to complex with members of the pRB family in order to induce flat cells when overexpressed in the transformed SW13 cell line.

To determine which interactions are functionally most significant, we used mutants of the E1A protein, each mutant capable of interacting with some but not all of the E1A-associated proteins. All of the E1A mutants used do not bind to pRB and p130. These mutants cotransfected with hBRG1 (either isoform) allowed a significant increase in flat-cell-inducing activity compared with hBRG1 cotransfected with wild-type E1A. These data indicate that both spliced forms of hBRG1 may greatly rely on their interaction with pRB and/or p130 for their flat-cell-inducing activity (for example, note the slight effect of E1A *dl*1104 on hBRG1's flat-cell activity).

Both hBRG1a and hBRM induce flat cells with similar potency, but when cotransfected with hBRM, the various mutants of E1A, regardless of their pRB family binding profile, eliminate flat-cell activity as strongly as wild-type E1A. This difference between hBRG1a and hBRM may be explained partially by referring to the two-hybrid binding data (Table 1), which suggests that hBRG1a has a stronger binding affinity for the pRB family and, unlike hBRM, can interact with p130. hBRM also demonstrates reduced affinity for pRB in vitro (Fig. 1B). All mutants of E1A used for this analysis (except *dl*1108) bind to p107 and not to pRB. Therefore, as demonstrated by its sensitivity to all of the E1A mutants used here, hBRM's flat-cell-inducing activity perhaps more greatly depends on its interaction with p107.

Another explanation for the difference between hBRG1 and hBRM may be related to the leaky binding of the E1A mutants to all of the pRB family. The binding profile of these mutants was determined by coimmunoprecipitating E1A with radiola-

beled cellular proteins (3, 4). Perhaps this analysis does not detect weak interactions between the E1A mutants and the pRB family members. Such weak interactions nevertheless may be strong enough to compete with hBRM, and not hBRG1, for binding to the pRB family, thus selectively eliminate hBRM's flat-cell activity. Finally, hBRM's flat-cell-inducing activity may require an additional, unidentified protein that is not required for hBRG1's activity. This unknown factor may be sequestered or functionally inactivated by either E1A or mutants of E1A that cannot bind to the pRB family. Therefore, aspects of hBRM function in SW13 cells may be pRB family independent.

One E1A mutant, *dl*1108, binds only to the E1A-associated protein p300. This mutant has the unexplained effect of eliminating the SW13 flat-cell activities of hBRM and both alternatively spliced forms of hBRG1. E1A *dl*1108 may exert an inhibitory effect on flat-cell induction by acting downstream of the hBRG1/hBRM-pRB family interaction. Neither hBRG1 nor hBRM interacts with the E1A-binding domain of p300 in the two-hybrid system (data not shown), and two other mutants used here, *dl*1107 and *dl*1109, also bind to p300 but do not eliminate hBRG1a's flat-cell activity. Nevertheless, the effect of *dl*1108 suggests a functional relationship between p300 and the hBRG1/hBRM family. Another possibility is that this mutant of E1A has an additional activity unrelated to its interaction with the E1A-associated proteins.

The regulation of E2F exemplifies how the pRB family functionally can interact with different members of another family of proteins. There are five known members of the E2F family, all demonstrating significant amino acid homology to one another. Nevertheless, pRB preferentially binds to E2Fs 1, 2, 3, and weakly to E2Fs 4 and 5. In contrast, p107 and p130 preferentially bind to E2Fs 4 and 5 (reviewed in reference 53). Similarly, the hBRG1/hBRM-pRB family interaction may represent a fine regulation of transcription by utilizing a multiplicity of possible interactions. The manner by which the pRB family regulates (or is regulated by) the hBRG1/hBRM family remains unknown. The hBRG1/hBRM family functions within a large complex of proteins (the human SWI/SNF complex) that likely alters the nucleosomal structure of chromatin (49). In so doing, the complex exposes DNA sequences recognized by specific transcription factors, allowing these factors to bind to promoter regions and perhaps activate gene expression (37). Interestingly, the LXCXE motif found in both hBRG1 and hBRM is not conserved in the SWI2/SNF2 homologs of lower eukaryotes. This modular addition may represent a unique hBRG1/hBRM function gained through evolution, further linking the cell cycle machinery to transcription via the pRB family. E2F is probably the most important activator of genes necessary for cell cycle progression. However, this fact does not preclude the possibility that the pRB family interacts with other regulators of transcription to tightly control gene expression. Indeed, the SWI2/SNF2 complex may assist the pRB family in its regulation of the E2F family. Cell cycle analysis of SW13 flat-cell induction and the biochemical characterization of the pRB-hBRG1/hBRM interaction perhaps will elucidate a novel regulatory mechanism of the pRB family.

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