RB is functionally distinct from its homologues in affecting glucocorticoid receptor mediated transcription and apoptosis

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Running Title: A Role for the N-terminal Domain of RB in GR Activities
SUMMARY

The cell cycle regulator, retinoblastoma protein, is known to potentiate glucocorticoid receptor activated transcription through the interaction of its pocket domain with the transcription coactivator, hBRM. We now show that glucocorticoid receptor induced apoptosis is also dependent on both the retinoblastoma protein and hBRM. p107 and p130, which share extensive sequence homology with the pocket domain of the retinoblastoma protein but not its amino terminal region, also interact with hBRM but do not support either glucocorticoid receptor dependent activity. This difference arises from the divergent N-terminal domain of the retinoblastoma protein which, when fused to the pocket domains, confers upon p107 and p130 the ability to influence glucocorticoid receptor activities. This effect probably results from the promotion of glucocorticoid receptor-targeted chromatin remodeling by the hBRM-containing SWI/SNF complex since the N-terminal domain of the retinoblastoma protein enhances glucocorticoid receptor-hBRM interactions. These results highlight that, besides the interaction between hBRM and the pocket domain of RB, the N-terminal region of the retinoblastoma protein is also essential for glucocorticoid receptor induced apoptosis and the potentiation of glucocorticoid receptor mediated transcription, and provide a basis for functional distinction between the retinoblastoma protein and its homologues.
INTRODUCTION

RB\(^1\) is a general tumor suppressor that was identified by virtue of a predisposition to childhood retinoblastoma when defects are present in its gene (1, 2). The tumor suppressing property of this 110 kDa nuclear phosphoprotein is associated with its central (A/B) and C-terminal (C) ‘pocket’ domains which were defined through their ability to bind cellular and viral proteins that affect the cell division cycle (reviewed in ref.3). These pocket domains are also present in p107 and p130, structural homologues of RB which exhibit similar protein binding and functional characteristics pertaining to the cell division cycle (4-7). Together, these homologues constitute the RB family of proteins and are linked by substantial direct evidence to regulatory roles in cell growth, differentiation and development (reviewed in ref.8). As a consequence of the similar pocket domains, these three homologues exhibit a noteworthy overlap of interacting proteins and \textit{in vivo} functionality (reviewed in ref.9), although they also bind differentially to other proteins (10, 11) and display distinct molecular and cellular characteristics.

One common characteristic is the ability to restrain cell growth by inhibiting the pocket domain-binding E2F family of transcription factors, these factors being crucial for the expression of genes that are vital for the S-phase of the cell division cycle. This repression of E2F activity and cell growth requires the participation of yet another pocket domain binding protein, hBRM (or its close homologue, BRG1) (12, 13).

hBRM and BRG1 are 200 kDa nuclear factors present in the large, multi-subunit human SWI/SNF complexes (14, 15) that contain eight or more proteins depending on the origin and state of the cell (16-18). These complexes function in reorganizing chromatin structure and
thus influence the activity of sequence-specific activator proteins by affecting their access to regulatory sites (16). In the repression of the S-phase inducing transcription factor E2F1, RB physically contacts both E2F1 and hBRM at the same time and thus targets the hBRM-containing SWI/SNF complex to E2F1 (12). In other cases, the SWI/SNF complex is targeted to specific promoters through interactions with DNA-binding components of the transcription machinery. A case in point is GR, a hormone activated transcription factor, that recruits the SWI/SNF complex through direct binding to the hBRM/BRG1 and stimulates nucleosome disruption at the glucocorticoid response element (GRE) (19, 20). Although the remodeling of the chromatin does not influence the ability of GR to access and occupy the GRE, it is essential for post-binding events involving the basic transcription machinery (20) since the TFIID component cannot access the relevant DNA sites until after treatment with glucocorticoid hormone (21). Hence, an abundance of hBRM can potentiate the transcriptional activity of the GR (14). Interestingly, this potentiation is further enhanced by the interaction of hBRM with RB (22).

The GR is a member of the nuclear hormone receptor superfamily, and consists of an N-terminal domain with a transcription activation function, a central DNA binding and dimerization domain, and a C-terminal ligand binding domain with an inducible transcription activation function (reviewed in ref.23). It binds as a homodimer to the canonical GRE, an imperfect palindrome, and accelerates transcription initiation at the promoter of the target genes. However, it functions as a monomer at composite GREs that are simultaneously occupied by other enhancer binding factors, or at tethering elements where it is tethered to the DNA by an interacting protein. At these complex sites, GR may function to
variously promote or repress gene expression (24), contingent on the context of the interacting proteins and DNA. The physiological outcome of these diverse molecular interactions is the regulation, by GR, of critical metabolic and developmental processes essential for survival (25, 26). At the cellular level, activation of the GR by glucocorticoids generally invokes antiproliferative effects, epitomized by the apoptosis of immature thymocytes (27) and various leukemic cell lines (28). It is because of these effects that glucocorticoids are employed as immunosuppressive, anti-inflammatory or cytostatic agents in the treatment of rheumatoid arthritis, collagen diseases, lymphatic leukemias, and lymphomas.

Developing on our earlier work, which showed that RB potentiates GR mediated transcription by interacting with hBRM (22), we queried if GR associated apoptosis was similarly influenced by RB and hBRM, and whether RB could be functionally replaced by its pocket domain homologues. We show here that both GR mediated transcription and apoptosis are dependent on RB and hBRM, but not p107 or p130. This is likely to result from the divergent N-terminal domain of RB that is unique in promoting the interaction of GR with hBRM, and hence the GR targeted chromatin remodeling activity of the SWI/SNF complex.
EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Interaction Analysis—Vectors and strains for the 2-hybrid system (provided by Dr. S. J. Elledge) were employed as previously described (29). The hBRM hybrid was constructed by inserting a flush-ended ClaI fragment of the hBRM cDNA into the NcoI and SmaI digested vector pACTII with flush ends. This created an in frame fusion of the coding sequence for the Gal4 activation domain (GAL4A) with that for amino acids D78 to I1389 of hBRM. Fusion of the RB pocket domain (N301-K928) with the Gal4 DNA binding domain (GAL4D) was achieved by inserting an RB cDNA fragment with a flush EcoRI end and a cohesive BamHI end into the pAS2 vector with a flush NcoI end and a cohesive BamHI end. The p107 pocket domain fusion (R337-H1068) was created by inserting a flush-ended BsiI/KpnI cDNA fragment into the SmaI digested vector pAS2, and the p130 pocket domain fusion (L322-H1139) was created by inserting a flush-ended XbaI fragment into pAS2 with flush NgoI ends. Double transfectants of S. cerevisiae strain Y190, verified for hybrid expression through immunoblotting, were analyzed for β-galactosidase activity as a measure of GAL1-lacZ expression resulting from the interaction between the GAL4A-hBRM hybrid and the GAL4D fusions.

Plasmids Expression vectors and cDNA for expressing mutant cyclin D1, RB, HA-RB*(C706F), p107, p130, GR, hBRM and E2F-1, and the reporter construct DHFRpro-CAT have been previously described (2, 4-7, 14, 22, 30-32). The GR mutant (R479D/D481R) was created w
the Transformer” Site-Directed Mutagenesis Kit (Clontech). The GRE-CAT reporter was created by placing a 180 bp synthetic DNA fragment carrying an appropriately positioned single GRE (20, 33) upstream of the coding sequence for CAT. The RBΔ(1-300) expression plasmid was created by replacing the RB coding sequence upstream of the unique EcoRI restriction site with the codon for methionine. The coding sequences for the chimeric proteins p107(1-384)RB(373-928), RB(1-372)p107(385-1058) and RB(1-372)p130(417-1139) were created via PCR fragment assembly. hBRM-HA-H6 was created with the mutagenesis kit to have the sequence YPYDVPDYAHAAAAHHHHH following the native C-terminus of hBRM. All constructions were verified by DNA sequence analysis.

**Cell Culture and Transfections**—All cells were grown as monolayers in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum under 5% CO2. Transfections were performed with 2 × 10^6 cells on 10 cm dishes, a total of 25 µg DNA and the Lipofactamine reagent (Gibco-BRL) for 5 hours in serum-free medium. Transfection efficiencies were monitored and normalized across experiments by including 0.5 µg of the constitutive RSV-luciferase reporter and analyzing for luciferase activity with the Promega kit. 48 hours after transfection, stable transfectants were selected with 400 µg/ml Gentamicin sulphate and/or 100 µg/ml Hygromycin B (Gibco-BRL) and well-isolated colonies were re-cloned twice through limiting dilutions before expanding the cultures. Where appropriate, GR was induced 24 hours after transfection by treating cells
with 10 nM dexamethasone for 24 hours (transactivation assays) or 48 hours (apoptosis assays).

Cell and nuclear extracts, immunoblotting and immunoprecipitation—Cells, or isolated nuclei (34), were first lysed in extraction buffer (22) and clarified. For immunoprecipitation, extracts, with 100 µg protein, were pre-cleared in extraction buffer with 5% (wt/vol) protein A-Sepharose beads for 30 minutes, then incubated with 5 µg of the appropriate antibodies for 2 hours at 4°C followed by 1 hour with 5% protein A-Sepharose beads. The beads were then washed extensively with extraction buffer before analysis.

For immunoblotting, equal amounts of protein were fractionated in each track by SDS-PAGE (35) and electroblotted onto nitrocellulose membranes. The membranes were blocked with phosphate buffered saline (PBS)/0.1% Tween-20/10% horse serum and incubated with antibodies for 1 hour at 4°C. After extensive washing with PBS/0.1% Tween-20, the retained antibodies were detected with Enhanced Chemiluminescene reagents (Amersham).

Antibodies specific for hBRM (H290) have been described before (22) and those specific for GR (P-20), RB (C-15), p107 (C-18), p130 (C-20), actin (I-19) and HA (F-7) were from Santa Cruz Biotechnology.

CAT and Caspase-3 Activity Assays—CAT activity was determined using both the chromatographic and the phase-extraction assays as previously described (34).
For caspase-3 activity, monolayers of cells on 15 cm dishes were harvested, processed and analyzed with the Fluorometric CaspACE™ Assay System (Promega) according to the manufacturers instructions.

For both activities, results from a minimum of three independent experiments, each performed in triplicate, were normalized to transfection efficiencies where necessary and reported as mean values with sample standard deviation error bars.
RESULTS

Establishment of the Glucocorticoid Responsive Test System—We first established a glucocorticoid responsive system that would enable us to selectively introduce the factors of interest. Several subclones of the cervical carcinoma cell line C33A were analyzed by northern blotting and immunoblotting, and were found not to express GR, full-length nuclear RB or hBRM, nor exhibit glucocorticoid receptor dependent gene expression as measured with the chloramphenicol acetyl transferase (CAT) cDNA placed downstream of a single GRE in the presence of the GR inducer, dexamethasone. These cells were then transfected with a neomycin selectable mammalian expression vector carrying the GRE-CAT fragment and a wild type or a dimerisation defective mutant GR cDNA downstream of the CMV promoter. Stable transfectants were screened for low-level expression of the receptor by immunoblotting and for the contiguity of the integrated GRE-CAT element by PCR analysis. The two clonal transfectants reported in this study, C33A/GR and C33A/GR*, exhibited approximately equivalent levels of wild type and mutant GR respectively but were still RB⁻/hBRM⁻. The mutant GR* carries two amino acid substitutions (R479D/D481R) in the dimerisation loop within the DNA binding domain which leaves it with little or no transactivation potential but capable of glucocorticoid induced transcriptional repression of specific genes (36). Accordingly, C33A/GR, but not C33A/GR*, was able to support expression of GRE-CAT when induced with 10 nM dexamethasone (Fig. 1A). A similar system was also established
with subclones of the osteosarcoma cell line SAOS-2 that were GR-/RB- but hBRM+.
However, in addition to the GR expression vector, these cells were also transfected with a
hygromycin selectable mammalian expression vector carrying a mutant cyclin D1 cDNA.
The expression of mutant cyclin D1 overcomes the growth arresting effects of RB
overexpression in SAOS-2 cells without other obvious effects (32). The stable double
transfectants reported here, SAOS-2 [cycD1mut/GR] and SAOS-2 [cycD1mut/GR*],
were confirmed to express low levels of GR or GR*, respectively, with no detectable
expression of hBRM or RB. The validity of using caspase-3 activity as a measure of
apoptosis in these cell lines was established by inducing cell death with etoposide,
stauroporin or paxlitaxel, and relating caspase-3 activity to the fragmentation of
chromatin as determined by DNA laddering assays and the appearance of sub-diploid
DNA content as determined by flow cytometry. With all three inducers, changes in
caspase-3 activity paralleled flow cytometry and DNA laddering data, having the added
benefit of high precision and reproducibility.

GR Dependent Apoptosis Requires RB and hBRM—Since GR mediated
transcription is enhanced by both RB and hBRM (14, 22) and GR has been shown to induce
apoptosis (28), we examined if GR induced apoptosis was similarly influenced. In order to
determine this, cells were transfected with expression vectors for wild type or mutant RB
and/or hBRM, allowed to recover for 24 hours and then treated with 10 nM
dexamethasone for 48 hours. Using C33A/GR cells, treatment with dexamethasone,
yielded no detectable apoptosis when RB or hBRM were present alone (Fig. 1B, lanes 1-
3). However, when RB and hBRM were both present, significant apoptosis was detected as evidenced by the almost 10 fold increase in caspase-3 activity (lane 4). When the interaction between RB and hBRM was abrogated by introducing hBRM with a defective LXCXE motif (lane 6) or RB with a pocket domain disabling mutation (lane 7), then apoptosis was not detected. The apoptosis detected here was clearly mediated by GR because it was not observed in the absence of the GR inducer dexamethasone (compare lanes 4 and 5) or with cells of the GR-negative parental line C33A (data not shown). These results suggest that GR mediated apoptosis requires the presence of both RB and hBRM and is dependent on their interaction through the pocket domain of RB and the LXCXE motif of hBRM.

Interestingly, the transfectant line C33A/GR*, which expresses the GR (R479D/D481R) double mutant incapable of dimerisation or transactivation, also yielded results similar to C33A/GR with respect to apoptosis. Specifically, dexamethasone treatment of C33A/GR* induced apoptosis but only when both functional RB and hBRM were also present (Fig. 1C). Hence, it can be inferred from these results, that GR mediated apoptosis is clearly independent of GR induced transcription. These conclusions were also borne out by results obtained with the osteosarcoma clones SAOS-2 [cycD1mut/GR] and SAOS-2 [cycD1mut/GR*] (Fig. 1D and E). The only difference that these cells yielded was that exogenous hBRM was not necessary for GR mediated apoptosis presumably because endogenous levels were sufficient. Taken together, these results show that GR mediated apoptosis is dependent on the presence and mutual interaction of RB and hBRM, but does not require GR mediated transcription.
p107 and p130 Interact with hBRM But Do Not Affect GR Dependent Apoptosis and Transcription—Since hBRM binds to the pocket domains of RB that are conserved in the RB homologues, p107 and p130, we investigated if hBRM could also interact with them. This was tested within the nuclear environment, where hBRM and the RB family members normally reside and function, using the yeast two-hybrid system (29). The bait for interaction was formed by the fusion of GAL4D with the pocket domains of RB, p107 and p130. These were co-expressed in S. cerevisiae strain Y190 with the prey hybrid, formed by fusing GAL4A to an almost full-length hBRM (D78-I1389) that carries the RB-binding LXCXE motif but lacks the bromo domain. Interactions between the hBRM and pocket domain fusions were assessed by indirect measurement of GAL1-lacZ expression. The results in Table I demonstrate that, similar to RB, pocket domain fusions of p107 and p130 are able to interact with hBRM, and that these interactions exhibit an avidity that is of the same order as the hBRM-RB pocket domain interaction. Our results here are slightly different from a similar study reported earlier where no interaction was detected between p130 and hBRM (37), but it should be noted that only the C-terminal portion of hBRM was used for interaction analysis in that study. The data in Table I also shows that this interaction is independent of the amino terminal regions of the RB family members as these are absent in the hybrids. This interaction was also assessed through co-immunoprecipitation of endogenous hBRM and RB family members from nuclear extracts of HeLa, a human cervical carcinoma line. When approximately a third of the hBRM was immunoprecipitated (Fig. 2A, row 1), about 15% of endogenous nuclear RB was also retrieved (row 2). In contrast, the transiently overexpressed HA-RB*(C706F)
mutant with a defective pocket domain was not detected in the immunoprecipitates (row 3), verifying the specificity of the assay. When the hBRM immunoprecipitates were analyzed for p107 and p130, about 10% of each was consistently detected (rows 4 and 5).

Since hBRM can also interact with p107 and p130, we next tested if these interactions were productive in affecting GR dependent transcription and apoptosis. SAOS-2 [cycD1mut/GR] cells were transfected with expression vectors for RB, p107 or p130, allowed to recover and then treated with 10 nM dexamethasone to induce the GR. When the extracts of cells treated for 24 hours were analyzed for GR mediated expression of GRE-CAT, it was clear that unlike RB (Fig. 2B, lane 2), neither p107 nor p130 affected this activity in any way (lanes 3 and 4). The abundant expression of all three members of the RB family was assessed in parallel experiments by co-transfecting with a vector for E2F-1 expression and the E2F-responsive reporter DHFRpro-CAT. The heterologous expression of RB, p107 or p130 reduced the E2F dependent expression of DHFRpro-CAT (Fig. 2C), indicating the overabundance of the respective proteins with functional pocket domains capable of binding and inhibiting the transcription factor E2F. When cells were treated with dexamethasone for 48 hours and analyzed for apoptosis by measuring caspase-3 activity in the cell extracts, a similar profile of effects was observed. Again, unlike RB (Fig. 2D, lane 2), neither p107 nor p130 (lanes 3 and 4) yielded any detectable apoptosis in the dexamethasone treated GR+ cells. Thus although p107 and p130 are capable of interacting with hBRM, unlike RB, they are incapable of affecting GR mediated transcription or apoptosis.
Requirement for the N-terminal Domain of RB—Although RB, p107 and p130 exhibit extensive homology over the C-terminal two-thirds where the pocket domains are located, RB differs significantly from p107 and p130 at the amino terminal regions (4-7). Since RB is the only member of this family that affects GR dependent transcription and apoptosis, we tested if the divergent N-terminal domain of RB could account for this distinguishing ability. Through a convenient deletion of the RB expression vector, we were able to express the mutant form RB\(\Delta(1-300)\) (Fig. 3A) that lacks the first 300 amino acids. This removes 80% of the divergent N-terminal region of RB. When RB\(\Delta(1-300)\) was expressed in SAOS-2 [cycD1mut/GR] cells, it was totally incapable of potentiating dexamethasone induced expression of the GRE-CAT reporter (Fig. 3C, lane 3). The presence of abundant protein with a functional pocket domain was confirmed by comparative immunoblotting (Fig. 3B, lane 3) and by the suppression of E2F dependent DHFR\(_{\text{pro}}\)-CAT expression in cells expressing RB\(\Delta(1-300)\) (Fig. 3D, lane 4). When these cells were analyzed for GR inducible apoptosis, it was clear that RB\(\Delta(1-300)\) could not support this activity either (Fig. 3E, lane 3). This clearly established that the N-terminal domain of RB is necessary for its effects on GR dependent apoptosis and transcription, but apparently dispensable for its regulation of E2F activity. In order to map the exact region within the 372-residue N-terminal domain responsible for the effects of RB on GR activities, we performed incremental deletions nested at either end of this region (Fig. 3A). After transfection of the relevant expression vectors into SAOS-2 [cycD1mut/GR] cells, these deletion mutants of RB were confirmed to be transiently expressed in abundance (Fig. 3B) and localized to the nucleus by immunofluorescent
analysis (data not shown). In dexamethasone treated cells, deletion of the first 20 N-terminal amino acid residues did not diminish the potentiation of GRE-CAT by RB, but instead yielded a slight, but consistent, enhancement of the potentiation (Fig. 3C, lane 4). However, deletion of the N-terminal 40 residues or the 20 residues preceding the pocket domain totally abrogated the transcription potentiating capability of RB (lanes 5-7). All the deletion mutants, however, retained the ability to suppress E2F activity (Fig. 3D), indicating that the pocket domains of these RB species are functionally viable. The profile of activities of the RB deletion mutants on GR dependent transcription was mirrored by their ability to support GR induced apoptosis, but with one difference. In this assay, all the deletion mutants tested failed to support GR induced apoptosis, including RBΔ(1-20) which exhibited an enhanced potentiation of GR dependent transcription (Fig. 3E). Similar results were obtained with C33A/GR when these mutants were co-expressed with hBRM (data not shown). Thus, these results clearly show that an intact N-terminal domain of RB is essential for GR induced apoptosis or for the potentiation of GR dependent transcription. Furthermore, the differential effects of RBΔ(1-20) indicate once again that GR induced apoptosis can be dissociated from GR mediated transcription.

The N-Terminal Domain of RB Functions With The Pocket Domains of p107 And p130—Since the N-terminal domain of RB is necessary for GR induced apoptosis and GR dependent transcription, we tested if the divergent N-terminal domains of p107 and p130 alone account for their inability to affect the GR activities. Through in vitro mutagenesis, the N-terminal domains of p107 and p130 were replaced with the
corresponding 372 residues from the N-terminal domain of RB to form RB(1-372)p107(385-1058) and RB(1-372)p130(417-1139) respectively (Fig. 4A). Similarly, the N-terminal region of RB was replaced with the corresponding sequence of p107 to form p107(1-384)RB(373-928). These chimeric molecules were then expressed in SAOS-2 [cycD1mut/GR] and verified for abundant nuclear expression and the possession of viable pocket domains by testing for their capability to suppress E2F activity (data not shown). When cells were treated with dexamethasone, the chimera p107(1-384)RB(373-928) exhibited no potentiation of GR mediated expression of GRE-CAT (Fig. 4B, lane 3). This indicated that the corresponding domain of a close homologue cannot functionally replace the N-terminal domain of RB. In contrast, the chimera formed by the N-terminal domain of RB and heterologous pocket domains, RB(1-372)p107(385-1058) and RB(1-372)p130(417-1139), achieved up to five fold potentiation of GR dependent transcription (lanes 5 and 7). Similar results were obtained for GR induced apoptosis in that p107(1-384)RB(373-928) failed to induce any apoptosis (Fig. 4C) whereas RB(1-372)p107(385-1058) and RB(1-372)p130(417-1139) clearly promoted cell death (lanes 5 and 7). These effects of RB(1-372)p107(385-1058), RB(1-372)p130(417-1139) and p107(1-384)RB(373-928) were also observed with C33A/GR cells transiently expressing hBRM (data not shown). However, in SAOS-2 [cycD1mut/GR*] and hBRM supplemented C33A/GR* cells which express mutant GR*, apoptosis but not transactivation was induced by the expression of RB(1-372)p107(385-1058) and RB(1-372)p130(417-1139) but not by p107(1-384)RB(373-928) (data not shown). This established that the N-terminal domain of RB possesses the ability to affect
GR dependent apoptosis and transcription, and also that this domain can confer p107 and p130 with the same capabilities when fused to their functional pocket domains.

*The N-terminal Domain of RB Enhances GR–hBRM Binding*—We next investigated the molecular basis for the effect of the N-terminal domain of RB on GR dependent apoptosis and transcription. Using the yeast 2-hybrid assay, we found that the N-terminal domain of RB did not directly interact with GR in the presence or absence of dexamethasone (data not shown) in agreement with a previous *in vitro* analysis (38). The screening of Gal4 activation domain fusion cDNA libraries derived from several human tissues, with a bait comprising the N-terminal domain of RB fused to the Gal4 DNA binding domain, also failed to identify any proteins that might interact with the N-terminal domain. Nevertheless, the possibility remained that the N-terminal domain of RB interacted with a factor(s) with too low an avidity to be detected by the screen.

We reasoned that if the N-terminal domain of RB did indeed interact with a factor(s) in affecting GR dependent apoptosis and transcription, then the presence of excess amounts of this domain should interfere with the normal function of RB. We introduced this by expressing the mutant RB*(C706F) which carries a non-functional pocket domain but an intact N-terminal domain. This would serve to provide excess N-terminal domain since RB* fails to achieve pocket domain dependent interactions (39). Using SAOS-2 [cycD1mut/GR] cells, the potentiation of GR mediated transcription was established with non-saturating amounts of RB (Fig. 5A, compare lanes 2 and 3), before increasing amounts of RB* were co-expressed. As expected, RB* itself failed to
potentiate GR mediated transcription (lane 4). As shown, the presence of increasing and excess amounts of RB* (lanes 5-7) also failed to affect the potentiation achieved by RB (lane 3). Similarly, the co-expression of the N-terminal domain alone with a nuclear localization signal and an epitope tag in excess amounts also failed to affect the potentiation of GR mediated transcription by RB (data not shown). This further suggests that the N-terminal domain of RB is unlikely to interact with other factors since excess amounts of this domain do not interfere with the effects of RB. This was also borne out by similar experiments focusing on GR induced apoptosis (Fig. 5B) where over expression of RB* failed to affect the RB dependent cell death (compare lanes 5-7 with lane 3). Similar results were obtained with C33A-H41 cells, derived from a clonal transfectant of C33A that stably expresses low levels of wild type GR (data not shown) and hBRM tagged at the C-terminus with a hemagglutinin (HA) epitope and a hexa-histidine motif (hBRM-HA-H6) (Fig. 5C, lanes 2 & 3). Taken together, this suggests that the N-terminal domain of RB does not, by itself, interact with other factors that are involved in the hBRM-dependent effects of RB on GR activities.

Since the N-terminal domain of RB functions in an hBRM dependent manner, we questioned whether it affected the interaction of GR and hBRM. C33A-H41 cells were transfected with expression vectors for RB and RBΔ(1-300), allowed to recover, and exposed to dexamethasone for 24 hours. Nuclear extracts prepared from these cells were then immunoprecipitated with HA specific antibodies to isolate the complex containing hBRM-HA-H6. The precipitate was then analyzed for hBRM-HA-H6, RB and GR. By immunoblotting with antibodies specific for the C-terminal portion of RB (Fig. 5D,
second panel), both full length RB (lane 3) and RBΔ(1-300) (lane 4) were detected in the hBRM-HA-H6 complex in approximately similar amounts, indicating that both species interacted equally well with hBRM. With GR specific antibodies (Fig. 5D, third panel), the amount of GR detected in the precipitate from untreated cells (lane 1) was increased by 11 fold in dexamethasone treated cells (lane 2), indicative of the expected ligand dependent interaction between the receptor and hBRM. However, when RB was transiently expressed, the amount of GR detected was increased by another five fold (lane 3). This was not observed with the expression of RBΔ(1-300) (lane 4). This analysis was repeated with the cell line C33A-H3 which expresses low levels of both hBRM-HA-H6 (Fig. 5C, lane 3) and the transcription defective, but repression and apoptosis capable double mutant GR*. When hBRM-HA-H6 immunoprecipitates were analyzed for the presence of GR* (Fig. 5D, fourth panel), it was clear that GR* also exhibited a ligand dependent interaction with hBRM (compare lanes 1 and 2). Interestingly, the presence of RB (lane 3), but not RBΔ(1-300) (lane 4), also increased the amount of co-immunoprecipitated GR* by almost 3 fold. These results demonstrate clearly that the presence of wild type RB significantly increases the stable incorporation of GR or GR* into the hBRM complex. In addition, the failure of the mutant RBΔ(1-300) to enhance ligand dependent receptor incorporation indicates that the N-terminal domain of RB is essential for this activity, although the pocket domain alone is sufficient for stable binding to hBRM. Thus, RB is likely to achieve its potentiation of GR mediated transcription, and the facilitation of GR and GR* dependent apoptosis, by enhancing the
incorporation of the nuclear hormone receptor into the hBRM complex. This capability, which is intimately linked to the divergent N-terminal domain of RB, functionally distinguishes it from the other RB family members whose N-terminal domains are more divergent.
DISCUSSION

Previous work has shown that the pocket domain of RB binds to the transcription coactivator hBRM, and that this interaction markedly potentiates GR mediated transcription (22). In the work reported here, we investigated if the other significant activity of GR, namely glucocorticoid-induced apoptosis, is also similarly affected by RB and hBRM; and if p107 and p130 are likewise potent modulators of GR activities. To address these questions in a meaningful way, a glucocorticoid responsive cell culture system was established by transfection to stably express wild type or mutant GR in initially glucocorticoid refractory RB− cells. This rendered the cells responsive to the GR inducer, dexamethasone, in terms of GR dependent apoptosis and transcription. The latter was assessed through a chromosomally integrated glucocorticoid responsive reporter system that likely existed in phased nucleosomes because the nucleosome disrupting factor, hBRM (16-18), was necessary for its expression. This mimics the response of endogenous glucocorticoid responsive genes within chromatin to the transcriptional activity of GR. A further validation of our system was provided by the observation that cells expressing GR*, incapable of transactivation, were susceptible to GR induced apoptosis. This is in accordance with other reports indicating that glucocorticoid induced apoptosis is independent of GR mediated transcription (40, 41).

The introduction of RB and/or hBRM into our RB−/hBRM− glucocorticoid sensitive system showed that the interaction between RB and hBRM is necessary for
glucocorticoid induced apoptosis, regardless of whether this is mediated by the wild type GR or the transactivation deficient GR*. However, neither p107 nor p130 affect the transactivation or apoptotic activities of GR, although both factors interact with hBRM via their pocket domains. Through deletion mutagenesis and domain swapping experiments, it became clear that the N-terminal domain of RB is unique amongst the RB family members in being able to affect GR mediated transcription and apoptosis. The clearest proof of this comes from the chimeric molecules, formed by the N-terminal domain of RB and the heterologous pocket domains of p107 and p130, which are competent in affecting GR activities. In line with an earlier report (38), we did not detect any direct interaction between RB and GR that might explain our observations. However, since hBRM is necessary for the effects of RB on the transactivation mediated by GR and the apoptosis induced by both GR and GR*, we focused on the previously documented GR-hBRM interaction (19). Indeed, we found that RB significantly enhances the amount of GR, or GR*, that stably associates with hBRM in the nucleus. Moreover, the N-terminal domain of RB is essential for this effect. Hence, the promotion and/or stabilization of the GR-hBRM interaction is likely to be the mechanism by which RB achieves its hBRM dependent effects on GR mediated transcription and apoptosis.

In the case of GR mediated transcription, the effect of RB is readily explained and is consistent with the current understanding of GR activity. Liganded GR can seek out and bind to its cognate response elements even if the latter are assembled into phased arrays of nucleosomes, but this binding does not lead to nucleosomal disruption (42) and the subsequent initiation of transcription. However, this occupation of DNA by the receptor
attracts the nucleosome disrupting activity of the hBRM-containing SWI/SNF complex (19, 20), through ligand dependent direct interaction between GR and hBRM (19). This targeted and reversible (43) nucleosomal disruption would enable other co-activators and the basic transcription machinery to access the relevant region of DNA template, resulting in transcription of the glucocorticoid responsive gene. Hence, the ability of RB to enhance the interaction of liganded GR with hBRM would in turn promote and sustain the disrupted form within the context of a reversible chromatin remodeling reaction, and consequently, increase GR mediated transcription. It is noteworthy that this model implicates the remodeling of the chromatin as the rate-limiting step in glucocorticoid induced gene expression.

Rationalizing the effects of RB and hBRM on GR induced apoptosis is somewhat less straightforward. The ability of both the wild type GR and the mutant GR* to effect apoptosis is consistent with earlier demonstrations that transactivation by the receptor is not necessary, and instead implicates the repressive functions of the GR (40, 41). This repression of gene expression by the GR is not well understood, due, in part, to the fact that most genes negatively regulated by GR do not exhibit the classical GRE within their cis regulatory elements. As such, various modes of action have been proposed for the repression of gene expression by GR (24). These invoke negative GRE which GR binds to achieve direct transrepression as in the case of the POMC gene (44), composite elements which GR binds together with other transcription factors as in the case of the proliferin gene (45), and tethering elements where GR itself does not bind to DNA but is recruited and secured there through interactions with a bound factor. The last is exemplified by the
transcription factor AP-1 dependent collagenase gene that is repressed by liganded GR (46, 47). Significantly, AP-1 activity is associated with cell proliferation and survival (48) and its repression by GR has been implicated in GR dependent apoptosis (40). Hence, it is altogether plausible that RB and hBRM affect GR dependent apoptosis by promoting the transrepressive activity of GR. It is already known that hBRM can function as a corepressor in partnership with RB, as evidenced by their role in inhibiting E2F1 activity (12). It is thus conceivable that GR, and GR*, achieve transcriptional repression by recruiting hBRM as a co-repressor to the regulatory site of the target gene. The presence of RB, which serves to enhance GR-hBRM interactions, would lead to a correspondingly stronger repression and that would in turn manifest as enhanced apoptosis.

The molecular mechanism by which RB enhances the incorporation of GR into the hBRM-containing complex is not clear, but our data indicates a crucial requirement for the N-terminal domain of RB in addition to the pocket domain. We did not detect any factors that might bind exclusively to the N-terminal domain in explanation of its role, and nor could we relate the potency of the domain to any structural element or motif. Instead, it is clear that the total structural information of the domain determines its efficacy since the entire N-terminal domain is necessary. It may participate in some very specific protein-protein interactions because it functions only when attached to a functional pocket domain that presumably brings it into the appropriate environment through interactions with pocket domain binding proteins. This environment might possibly involve the other members of the chromatin remodeling, and hBRM-containing, SWI/SNF complex with which RB would be closely associated when bound to hBRM.
This interaction might possibly induce or stabilize a conformational state conducive to the function of the SWI/SNF complex. Alternatively, RB may function as a bridge to tether other RB-binding factors to the hBRM complex, analogous to its ability to bind E2F1 and hBRM at the same time (12). Yet another possibility arises from the fact that the N-terminal domain of RB participates in its oligomerisation (49), and it may thus function as a multimeric bridge to tether pocket domain binding factors to the hBRM complex.

In contrast to RB, neither p107 nor p130 exhibited the capacity to affect GR mediated transcription or apoptosis. This deficiency arises from their more divergent N-terminal domains (4-7). This is aptly demonstrated by the ability of the N-terminal domain of RB to confer the pocket domains of p107 and p130 with the ability to affect GR dependent transcription and apoptosis. Presumably, the N-terminal domains of p107 and p130 are involved in yet unascertained molecular events that would set these factors apart from RB with respect to cellular or gross physiological function. Investigations to date have often highlighted the similarities in the molecular properties of the RB family members, with differences being restricted to subtle specifications in interaction properties or cellular effects. This report provides a basis for functional distinction between RB and its homologues p107 and p130 based on their differential modulation of GR activities, and defines the structural basis for this distinction.

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FOOTNOTES

1. Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; GAL4A, Gal4 activation domain; GAL4D, Gal4 DNA binding domain; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HA, hemagglutinin; RB, retinoblastoma protein.
FIGURE LEGENDS

FIG. 1. **Effect of RB and hBRM on GR induced apoptosis.** (A) C33A/GR which expresses wild type GR, but not C33A/GR* which expresses the dimerisation and transactivation defective mutant GR*, exhibits glucocorticoid responsive transcriptional activation. (B–E) Glucocorticoid induced apoptosis requires RB and hBRM but not GR mediated transcription. 48 hr after dexamethasone treatment of cells transfected with the indicated proteins, apoptosis was assessed by measuring caspase-3 activity in the extracts of C33A/GR, C33A/GR*, SAOS-2 [cycD1mut/GR] and SAOS-2 [cycD1mut/GR].

FIG. 2. **All members of the RB family interact with hBRM, but only RB supports GR mediated transcription and apoptosis.** (A) hBRM was immunoprecipitated from nuclear extracts of untransfected (rows 1, 2, 4, 5) or HA-RB*(C706F) overexpressing (row 3) HeLa cells and the co-retrieval of associated proteins assessed by immunoblot analysis. (B–D) Extracts of SAOS-2 [cycD1mut/GR] cells transiently expressing the indicated factors were treated with dexamethasone and analyzed for GR mediated transcription by measuring the CAT activity resulting from the expression of the chromosomally integrated GR responsive reporter construct GRE-CAT, the functional viability of the transfected RB family proteins through their repression of E2F activity as reflected by the expression of the E2F responsive construct DHFRpro-CAT, and the extent of glucocorticoid induced apoptosis as measured by caspase-3 activity.
FIG. 3. The N-terminal domain of RB is necessary for GR/GR* induced apoptosis and GR mediated transcription. (A) Schematic representation of the RB mutants created by deleting various parts of the N-terminal domain. (B) The expression of the RB mutants in transiently transfected SAOS-2 [cycD1mut/GR] cells was assessed through immunoblot analysis of nuclear extracts with antibodies specific for the C-pocket of RB. (C) The effect of the RB mutants on GR mediated transcription was determined by measuring the expression of the GR responsive reporter GRE-CAT. (D) The viability of the pocket domains of the RB mutants was demonstrated by their ability to inhibit E2F activity as measured by the expression of DHFR_pro-CAT. (E) Glucocorticoid induced apoptosis in the presence of the RB mutants was determined by measuring caspase-3 activity.

FIG. 4. The N-terminal domain of RB renders the pocket domains of p107 and p130 capable of supporting GR induced apoptosis and transcription. (A) Schematic representation of the chimeric molecules created by shuffling the N-terminal domains of RB, p107 and p130. (B) Total extracts of cells transiently expressing the wild type or chimeric molecules were analyzed for the expression of the co-transfected GRE-CAT as a reflection of GR mediated transcription. (C) The extent of apoptosis after 48 hr of dexamethasone treatment of the transfected cells was gauged by the caspase-3 activity.

FIG. 5. The N-terminal domain of RB does not independently recruit or exclude factors, but enhances the interaction of GR and GR* with hBRM. (A) GR mediated transcription, and (B) GR induced apoptosis in dexamethasone treated SAOS-2 [cycD1mut/GR] cells...
expressing sub-optimal levels of RB and increasing amounts of the pocket domain mutant RB*. (C) Cloned C33A transfectants H41 and H3, expressing GR and GR* respectively, were confirmed to express low levels of hBRM-HA-H6 through comparative immunoblotting of total cell extracts from the indicated cell lines with hBRM specific antibodies. Actin specific antibodies indicate that comparable total protein was analyzed. (D) The effect of RB and RBΔ(1-300) on the incorporation of GR into the hBRM complex. C33A-H41 and C33A-H3 cells were transfected as indicated and treated with dexamethasone. Proteins complexes immunoprecipitated with HA-epitope specific antibodies from total cell extracts were then analyzed by immunoblot analysis with antibodies against hBRM, RB and GR.
TABLE I

Interaction of hBRM with members of the RB family

Double-transfected yeast cells in the two-hybrid system were analyzed for β-galactosidase activity as a measure of GAL1-lacZ expression resulting from the interaction between the GAL4A-hBRM hybrid and the GAL4D fusions. The values are expressed relative to the GAL4D/GAL4A double-transfectant.

<table>
<thead>
<tr>
<th>GAL4 activation-domain fusions</th>
<th>DNA-binding-domain fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAL4D</td>
</tr>
<tr>
<td>GAL4A</td>
<td>1</td>
</tr>
<tr>
<td>GAL4A-hBRM</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>
Figure 1

A) GRE-CAT

B) C33A/GR

C) C33A/GR*

D) SAOS-2 [cycD1mut/GR]

E) SAOS-2 [cycD1mut/GR*]
Figure 2

A. Nuclear extract (1/3) hBRM immunoprecipitate

- hBRM
- RB
- HA-RB*(C706F)
- p107
- p130

B. GRE-CAT

<table>
<thead>
<tr>
<th>Vector</th>
<th>RB</th>
<th>p107</th>
<th>p130</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
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<tr>
<td>2</td>
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<td></td>
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<td>4</td>
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</tbody>
</table>

C. DHFR\textsubscript{pro}-CAT

D. Apoptosis

- Relative Caspase-3 Activity
- Relative CAT Activity
Figure 3

A

N-terminal domain 'A/B' Pocket 'C' Pocket

RB
RBΔ(1-300)
RBΔ(1-20)
RBΔ(1-40)
RbΔ(351-372)
RBΔ(331-372)

B

Vector RB RB Δ(331-372) RB Δ(1-300) RB Δ(1-20) RB Δ(1-40) RB Δ(351-372) RB Δ(331-372)

C

GRE-CAT

D

DHFRpro-CAT

E

Apoptosis

0 2 4 6

0 2 4 6

0 2 4 6

E2F-1 − + + + + + +

Vector RB RB Δ(1-300) RB Δ(1-20) RB Δ(1-40) RB Δ(351-372) RB Δ(331-372)
**Figure 4**

**Panel A**

Box diagrams illustrate the N-terminal domain and the 'A/B' Pocket and 'C' Pocket domains of RB, p107, and p130. The domains are shown as bars with the 'A/B' Pocket and 'C' Pocket indicated by different shading.

**Panel B**

**Gremlin-CAT (GRE-CAT)**

A bar graph showing relative CAT activity with error bars. The x-axis represents different constructs, including Vector, RB, p107(1-384)RB(373-928), p107(1-372)p107(385-1058), RB(1-372)p130(417-1139), p130(417-1139), and p130(417-1139).

**Panel C**

**Apoptosis**

Figure 5

A. GRE-CAT

Relative CAT Activity

RB 1 2 3 4 5 6 7
RB* - ++ + - ++ +

B. Apoptosis

Relative Caspase Activity

RB 1 2 3 4 5 6 7
RB* - -- ++ +++

C. C33A-H41 (GR) C33A-H3 (GR*)

D. Vector Vector RB RBΔ(1-300)

Dex: - + + +

hBRM-HA-H6

C33A-H41

RB

RBΔ(1-300)

GR

GR*

anti-hBRM

anti-actin

1 2 3 4 5 6
RB is functionally distinct from its homologues in affecting glucocorticoid receptor mediated transcription and apoptosis
Paramjeet Singh, Siew Wee Chan and Wanjin Hong

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