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The extreme C terminus of progesterone receptor contains a transcriptional repressor domain that functions through a putative corepressor

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ABSTRACT **Binding of a hormone agonist to a steroid receptor leads to the dissociation of heat shock proteins, dimerization, specific DNA binding, and target gene activation. Although the progesterone antagonist RU486 can induce most of these events, it fails to activate human progesterone receptor (hPR)-dependent transcription. We have previously demonstrated that a conformational change is a key event leading to receptor activation. The major conformational distinction between hormone- and antihormone-bound receptors occurs within the C-terminal portion of the molecule. Furthermore, hPR mutants lacking the C terminus become transcriptionally active in the presence of RU486. These results suggest that the C terminus contains a repressor domain that inhibits the transcriptional activity of the RU486-bound hPR. In this study, we have defined a 12 amino acid (12AA) region in the C terminus of hPR that is necessary and sufficient for the repressor function when fused to the C-terminal truncated hPR or to the GAL4 DNA-binding domain. Mutations in the 12AA domain (aa 917–928) generate an hPR that is active in the presence of RU486. Furthermore, overexpression of the 12AA peptide activates the RU486-bound wild-type hPR without affecting progesterone-dependent activation. These results suggest that association of the 12AA repressor region with a corepressor might inactivate hPR activity when it is bound to RU486. We propose that binding of a hormone agonist to the receptor changes its conformation in the ligand-binding domain so that association with coactivator is promoted and activation of target gene occurs.**

The steroids, retinoids, thyroid hormones, and vitamin D_3 regulate important biological events including reproduction, development, differentiation, homeostasis, and behavior. Members of the nuclear receptor superfamily, which are ligand-dependent intracellular transcription factors, are responsible for mediating these signals (1–4). Formation of the steroid hormone-receptor complex induces dissociation of heat shock proteins, receptor dimerization, DNA binding, and gene activation. It has been shown that heat shock protein dissociation, dimerization, and DNA-binding events are not sufficient for the progesterone receptor (PR) to activate target genes (5–9). Our previous results demonstrated that proper conformational modification induced by ligand is necessary for the receptor activation. As determined by limited proteolytic digestion and monoclonal antibody-epitope mapping, progestin treatment induces a dramatic conformational change, rendering the entire ligand-binding domain (LBD) resistant to protease digestion and antibody recognition (8, 10, 11). Similar conformational changes have also been observed in estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), thyroid receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), and retinoic X receptor (RXR) (9, 12–16). In contrast, hormonal antagonists including RU486 induce a distinct structural alteration in the LBD. The conformational distinction induced by RU486 centers upon the final 3-kDa region of the C terminus that is freely accessible for protease and antibody recognition (8, 10). This result is generally consistent with the recently available crystal structure information for unliganded RXR and liganded TR and RAR (17–19). Therefore, the C-terminal portion of PR must play a role in distinguishing between agonist and antagonist activity.

More direct evidence for the repressor function in the C-terminal tails of human PR (hPR) and GR is obtained from studying receptor mutants. PR mutants lacking the C-terminal 42 or 54 amino acids can be specifically activated by progesterone antagonists, such as RU486, although they are unable to bind progesterone agonists (10). Similarly, a GR mutant containing point mutations at amino acids 770 and 771 and deletion of amino acids 780 and 781 in the C-terminal tail also results in a receptor that can only be activated by the antagonist (20). Based on these results, it has been proposed that the C-terminal tail of steroid hormone receptor may contain a repressor function that represses the activity of unliganded or antagonist-occupied steroid receptor (1, 10). However, the precise location of the repressor domain and the mechanism of the repression function remain uncharacterized.

In this study, we have analyzed multiple mutants of hPR type B (hPRB) and defined a 12 amino acid region (residues 917–928) in the C-terminal tail of hPRB that is responsible for the repression function. The repressor region has active silencer activity when fused to the GAL4 DNA-binding domain. Furthermore, overexpression of the repressor region can enhance the antagonist RU486-dependent activation of the wild-type hPR without affecting the progesterone-dependent activity. These results suggest that the repressor region of hPR functions through interaction with a soluble intracellular corepressor. This corepressor interacts with the RU486-occupied receptor to repress the target gene transcription. Removal of this inhibitory factor from the RU486-receptor complex leads to partial recovery of its transcription activity even in the absence of an agonist.

MATERIALS AND METHODS

hPRB Mutants. A mammalian expression vector pRShPRB was constructed by blunt ligation of a cDNA coding for the full-length hPRB from pT7β*Sal*/*Stu*-hPRB plasmid (8) and a vector backbone from pRShGRα plasmid (21). The hPRB

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Abbreviations: PR, progesterone receptor; hPR, human PR; hPRB, hPR type B; GR, glucocorticoid receptor; AR, androgen receptor; RAR, retinoic acid receptor; TR, thyroid receptor; DBD, DNA-binding domain; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; LBD, ligand-binding domain; 12AA, 12 amino acid; GLVP, a chimeric regulator containing GAL4 DBD, PR LBD, and VP16 activation domain; SMRT, silencing mediator for RAR and TR.
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cDNA was released from pT7β*Sal*/*Stu* plasmid by *Hin*dIII (partial) and *Eco*RI digestion. The vector backbone was obtained by Asp-718 and *Bam*HI digestion. In the resulting vector, pRShPRB, the transcription is controlled by an Rous sarcoma virus-long terminal repeat promoter. The C-terminal deletion mutants of hPRB were constructed in the same vector backbone by replacing a 3′ cDNA fragment (*Hin*dIII/*Xba*I, ≈390 bp) with PCR-amplified fragments bearing serial 3′ deletions. PCR was carried out by using wild-type hPRB cDNA as template, *pfu* DNA polymerase (Stratagene), and a common 5′ primer containing internal *Hin*dIII site (underlined), 5′-GTCAAGCTTCAAGTTAGCCAAGAAGAGTTCCTCT, and a 3′ primer corresponding to a specific mutant. An in frame stop codon and an *Xba*I recognition sequence were incorporated in all 3′ primers for precise stops of translation and convenient subcloning. All cDNA fragments synthesized by PCR were confirmed by sequence analyses before cloning into expression vectors.

Expression vectors pBShPRB-891/917-928 and pBShPRB-879/903-933 were constructed by PCR using a primer-directed deletion method as described previously (22). The 5′ primer was the common one corresponding to the internal *Hin*dIII site as described above. The primer for deleting amino acid residues 892–916 in mutant hPRB891/917-928 was 5′-AAGGGGTTTCACCATCCCTGCCAATATCTTGGGC-AAGCAGTACAGATGAAGTTG, which was overlapping with a 3′ primer, 5′-TAATCTAGATATCAAAGGGGTTT-CACCATCCCTGC. This 3′ primer had a stop codon after amino acid residue 928. The complementary PCR primer pair for deleting amino acid residues 880–902 in mutant hPRB-879/903–933 were 5′-CTTACAAAACTTCTTGATAACGT-TGAATTTCCAGAAATGATG and its antisense strand. A 3′ primer used to cover the complete C terminus was 5′-TAATCTAGATATCTCACTTTTTATGAAAGAGAAG. The fragments (*Hin*dIII/*Xba*I) amplified by PCR were cloned, sequenced, and then used to replace the 3′ corresponding portion of the wild-type hPRB cDNA.

The cDNA insert in vector pRShPRB(m) encodes a hPRB mutant bearing three amino acid changes (I920E, L921A, and G923E). The site mutations were generated by a pair of mismatched complementary PCR primers, 5′-TTACCCAAG-GAAGCGGCAGAGATGGTGAA (changed bases are underlined) and its antisense strand, as described (23).

To construct mammalian expression vectors pABGAL4-12 amino acid region (12AA) and pABGAL4-12AA(m) (Fig. 3), two pairs of complementary oligonucleotides, 5′-TCGACT-TACCCAAGATATTGGCAGGGATGGTGAAACCCCT-T*TAG*/5′-GATCC*TA*AAGGGGTTTCACCATCCCTGC-CAATATCTTGGGTAAG and 5′-TCGACTTACCCAAG-GAAGCGGCAGAGATGGTGAAACCCCTT*TAG*/5′-GATCC*TA*AAGGGGTTTCACCATCTCTGCCGCTTCCT-GGGTAAG (stop codons are in italics and changed bases are underlined), were annealed and inserted in frame into the *Sal*I/*Bam*HI sites of plasmid pABGAL (24), respectively. The resulted vectors express chimeric proteins containing GAL4 DNA-binding domain (DBD, residues 1–147) and either the amino acid residues 917–928 in hPRB or its mutant form bearing the same three amino acid changes as in the pRSh-PRB(m) described above.

Transfections. Transient transfections of Lmtk$^-$ cells by polybrene method and chloramphenicol acetyltransferase (CAT) assays were performed as described (25). The HeLa cells (10^6) in 100-mm dishes were incubated overnight in DMEM containing 5% charcoal-stripped fetal bovine serum, and were transfected by the calcium phosphate method described (26). Briefly, cells were incubated for 20 hr with plasmid DNAs, and then treated for 24 hr with or without hormones in fresh medium before harvesting. Reporter plasmids including PRE2-TATA-CAT, PRE2-TK-CAT, 17×4-TATA-CAT, and 17×4-TK-CAT have been described previously (10, 27, 28).

RESULTS

Amino Acids 917–928 in hPRB Inhibit RU486-Dependent Transcriptional Activation. Our previous studies indicate that the progesterone antagonist RU486, when bound to receptor, induces heat shock protein dissociation, receptor dimerization, and specific DNA binding without activating the wild-type PR (8, 10). In contrast, a PR mutant lacking the C-terminal 42 amino acids exhibits RU486-dependent transcriptional activity (10). These results suggest that a repressor function that inhibits the activation of the RU486-bound PR may exist in the C-terminal region of PR. To identify the repressor domain, we generated a series of C-terminal deletion constructs from the wild-type hPRB. These mutants were analyzed for their RU486-stimulated transcriptional activities by cotransfection

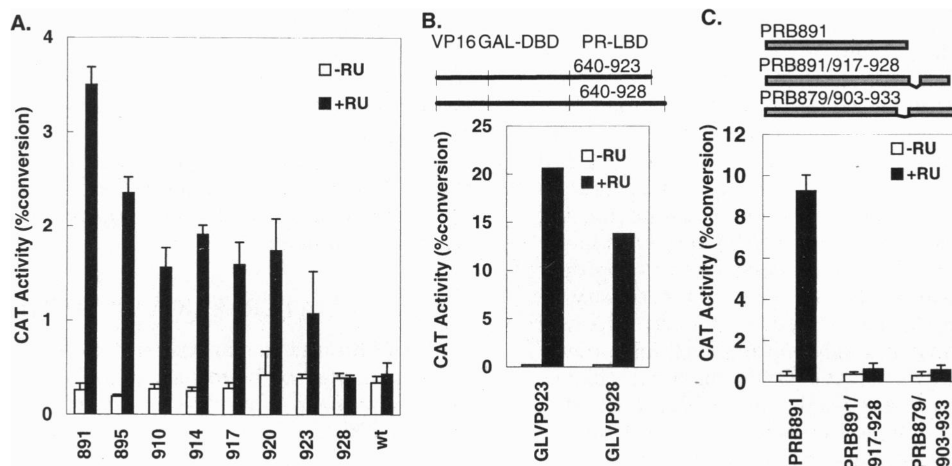

FIG. 1. Characterization of the repressor domain in the C terminus of hPRB. (*A*) Transcriptional activation of C-terminal hPRB deletion mutants by RU486. HeLa cells were transiently transfected with 5 μg of pRShPRB plasmids or its derivatives containing hPRB C-terminal deletion mutants as indicated and 5 μg of PRE2-TATA-CAT reporter plasmids. Transfected cells were treated with RU486 (10^{-8} M) or solvent only for 24 hr before CAT assay. (*B*) Mutants hPRB923 and 928 bind RU486. HeLa cells were transfected with GLVP923 or GLVP928 (*Upper*) and 17-mer-TATA-CAT reporter, and treated with or without RU486. VP16, activation domain of VP16 protein. GAL-DBD, GAL4 DNA-binding domain. PR-LBD, PR ligand-binding domain. (*C*) The C-terminal region of hPRB contains a repressor domain. HeLa cells were transfected with indicated plasmids and PRE2-TATA-CAT reporter plasmids as in *A*.

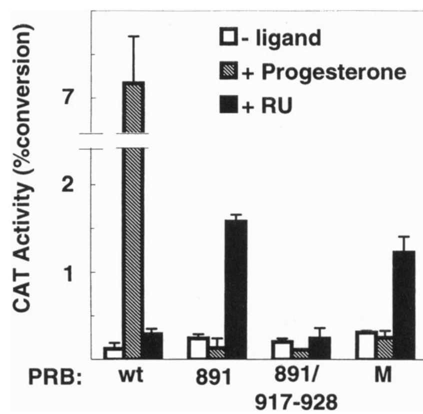

FIG. 2. Mutation in the 12AA region of hPRB abolishes its repressor activity. HeLa cells were transfected with expression vectors bearing indicated hPRB mutants and PRE2-TATA-CAT reporter plasmids as in Fig. 1, and treated with solvent only, 10^{-8} M progesterone or RU486 for 24 hr before CAT assay. PRB(M), a full-length mutant bearing three amino acid changes (I920E, L921A, and G923E).

into HeLa cells with a PRE2-TATA-CAT reporter construct. As expected, the wild-type PR and all eight deletion mutants were unable to activate target gene transcription in the absence of ligand (Fig. 1*A*). Also, in the presence of progesterone agonist, all of the mutants were unable to activate the reporter gene transcription (data not shown). This is due to the inability of these mutants to bind to progesterone. In the presence of RU486, six of the C-terminal deletion mutants, including hPRB-891, -895, -910, -914, -917, and -920, exhibited RU486-dependent transcriptional activities. However, the mutant hPRB-923 missing the final C-terminal 10 amino acids had only a moderate activity in the presence of RU486. Furthermore, hPRB-928 mutant, in which only five amino acids were deleted from the C terminus, had no RU486-dependent activity, nor does the wild-type hPRB (Fig. 1*A*). Thus, the repressor domain that inhibits the activity of RU486-receptor complex must reside primarily in the region lying N-terminal to amino acid 928.

To substantiate this conclusion, it is important to rule out the possibilities that alteration of the transcriptional activities of mutants hPRB-923 and -928 was due to the inability of these mutants to bind RU486, to dimerize, or to bind DNA. To test these possibilities, we constructed two chimeric regulators consisting of the VP16 activation domain fused to the GAL4 DBD and LBDs of these two hPRB mutants. Both regulators were transcriptionally active only in the presence of RU486 (Fig. 1*B*). These results indicate that RU486 can still bind to these mutants, and induce dimerization and DNA binding. Taken together, we conclude that the C-terminal boundary of the repressor region must be located between amino acid 923 and 928.

To map the N-terminal boundary of the repressor region, we took advantage of our previous work which demonstrated that C-terminal deletion mutants, hPRB-891 and -879, were transcriptionally active upon binding to RU486, due to the removal of the C-terminal repression function (refs. 1 and 10 and Fig. 1*C*). Thus, it should be possible to fuse a minimum region that contains the repressor function to hPRB-891 or -879 to repress the RU486-dependent activation. We fused fragments containing residues 903–933 and 917–928 to the C-terminal ends of mutants hPRB-879 and -891, respectively, and analyzed their activities in a transfection assay in the presence of RU486 or progesterone. Fig. 1*C* shows that fusion of either a 12AA fragment containing residues 917–928 to the mutant hPRB-891, or a larger fragment (residues 903–933) including the 12AA region to the mutant hPRB-879, completely inhibited the RU486-dependent activities. These results are in contrast to our earlier finding that fusion of an unrelated 12 amino acid peptide to the C-terminal end of the mutant hPRB-879 has no effect on RU486 binding or on its transcriptional activity (10). These results also substantiate that the lack of transcriptional activities of mutants hPRB-891/917-928 and -879/903-933 are not due to their inability to bind RU486. In summary, our results clearly indicate that the 12AA region (residues 917–928) in the C terminus is sufficient to repress the RU486-dependent transcriptional activity of the receptor.

Mutations in the 12AA Repressor Region Induce the hPRB Activity in the Presence of RU486. Since the 12AA region in the C terminus of hPRB can repress the activation of RU486-bound hPRB-891 mutant, we asked whether disruption of the repressor function located in the 12AA region can convert the full-length PR into a form that can be activated by RU486. Therefore, we generated a full-length mutant that contained only three amino acid changes (I920E, L921A, and G923E) in the 12AA region. These three amino acids were chosen because of their relative conservation among steroid receptors including PR, GR, AR, and mineralocorticoid receptor. Unlike wild-type hPRB and mutant hPRB891/917-928 that could not be activated by RU486, the full-length hPRB mutant

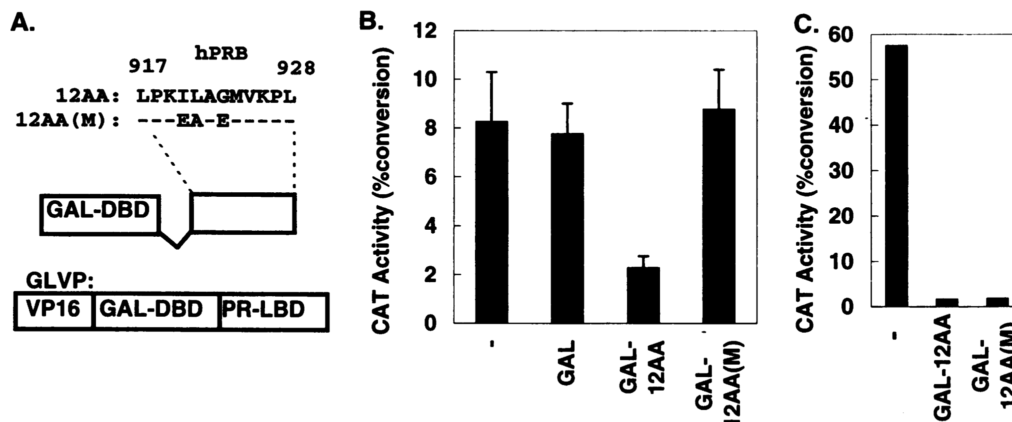

FIG. 3. The 12AA region has the ability to repress TK promoter. (*A*) Schematic illustration of fusion proteins, GAL4-DBD-12AA, GAL4-DBD-12AA(m), and GLVP. The 12AA wild-type sequence in hPRB and the three amino acid changes in its mutant form 12AA(m) are indicated. (*B*) Transfection analyses. Lmtk$^-$ cells were transiently transfected by polybrene method with 5 μg of indicated plasmids, where GAL means GAL4-DBD, and 5 μg of 17×4-TK-CAT reporter plasmids. The transfected cells were incubated for 40 hr before harvest. CAT assays were performed as described. (*C*) Both GAL-12AA and GAL-12AA(M) proteins are efficiently expressed. Cells were cotransfected with 5 μg of indicated plasmids plus 1 μg GLVP and 5 μg 17 mer-TATA-CAT plasmids. The transfected cells were treated with progesterone (10^{-8} M) for 24 hr before CAT assay.

containing three amino acid changes was significantly activated by RU486 to a level similar to that exhibited by the 3′ deletion mutant, hPRB-891 (Fig. 2). These results suggest that the three amino acids located in the 12AA region are essential for hPR repressor function. Disruption of the repressor function by the three amino acid mutations allows a full-length receptor to be activated by RU486.

The Repressor Function of the 12AA Region in hPRB Is Transferable. After defining the 12AA repressor region in the C terminus of PR, we asked whether the repressor region can function by itself or only in the context of PR. For this reason, we fused fragments containing either the 12AA or its mutant form 12AA(m) bearing the same three amino acid changes as described above in the hPRB(m) mutant to GAL4-DBD (Fig. 3*A*). These two chimeric constructs were analyzed by cotransfection with a 17 × 4- thymidine kinase (TK)-CAT reporter in mouse fibroblast Lmtk$^-$ cells in which the TK promoter exhibited higher basal level of activity. As demonstrated previously, expression of the GAL4-DBD had little effect on the TK promoter activity (refs. 24 and 29 and Fig. 3*B*). In contrast, the 12AA fragment fused to the GAL4-DBD significantly repressed reporter activity from the TK-promoter, while the three amino acid mutations (Fig. 3*A*) completely abolished the repressor activity (Fig. 3*B*). These results indicate that the 12AA peptide has an intrinsic repressor activity and can function when fused to a heterologous DNA binding domain. In addition, it further confirms that the three amino acids (I920, L921, and G923) in the 12AA region of hPRB are essential for its repressor function.

To ensure that both GAL-12AA and -12AA(m) were efficiently expressed and recruited to the promoter DNA in the reporter construct by the GAL4-DBD, we performed a competition assay in Lmtk$^-$ cells by cotransfection of the GAL4-DBD-12AA or -12AA(m) with the 17×4-TATA-CAT reporter and a chimeric regulator, GLVP933 (Fig. 3*A*), containing the VP16 activation domain fused to the GAL4-DBD and the entire LBD of hPRB. Without GAL4-DBD-12AA or -12AA(m) competitors, the GLVP933 strongly stimulated the reporter activity in the presence of progesterone. In contrast, cotransfection of either the GAL4-DBD-12AA or -12AA(m) with the GLVP at a 5:1 ratio significantly inhibited the reporter expression due to competition for binding to the 17-mer GAL4-binding sequence in the reporter construct (Fig. 3*C*). These results indicate that both GAL4-DBD-12AA and -12AA(m) were efficiently expressed. Therefore, the lack of repressor function by the mutated 12AA fragment is not due to its low expression or lack of DNA binding ability.

Overexpression of the 12AA Repressor Region Enhances RU486-Dependent Activity of PR in Cells. Since the 12AA repressor region appears to function through an intermolecular mechanism, we further asked whether the repression is through direct interaction with the basal transcriptional machinery, or requires an inhibitory cofactor, such as corepressor. To address this question, we overexpressed the GAL4-DBD-12AA together with wild-type hPRB in the presence of RU486 to determine whether the 12AA has the ability to squelch the corepressor from the RU486-bound hPRB, and thus activate RU486-dependent transcription by PR. In the presence of RU486, overexpression of the GAL4-DBD-12AA significantly increased the reporter activity in a dose-dependent manner, while overexpression of GAL4-DBD-12AA(m) under the same conditions did not affect the reporter expression (Fig. 4*A*). Thus it is likely that in the presence of progesterone, the PR is no longer able to bind to the putative corepressor. These results also suggest that the 12AA repressor region located in the C terminus of hPRB serves as an interactive domain for a corepressor. The association of this coregulator with the RU486-bound PR renders the receptor to a transcriptionally inactive form. In the presence of progesterone, neither the GAL4-DBD-12AA nor -12AA(m) affected the transcriptional activity of the wild-type hPRB (Fig. 4*B*).

DISCUSSION

Investigations of the inhibitory mechanism by which hormone antagonists act have provided valuable information for understanding the receptor activation process. The inability of RU486 to activate PR indicates that recognition of a PRE alone is not sufficient for the receptor to be active (ref. 10 and Fig. 1). Using protease digestion and antibody mapping, it has been demonstrated that progesterone and RU486 induce different conformational changes in the PR (8, 10, 11). The major distinction between these conformation changes is at the C-terminal 30–40 amino acids of the PR. Deletion of this C-terminal region creates a mutant PR that responds positively to RU486 activation (10). The RU486-induced transcriptional activity is due primarily to AF-1 activity in the N-terminal domain of the molecule (unpublished data). Our results strongly argue that the rearrangements of the C-terminal tail induced by agonist or antagonist determine the activation function of the receptor. We have proposed that there is a repressor function located in the C-terminal portion that maintains the RU486-bound receptor silent. In this report, we mapped this repression function to a 12AA region containing amino acid residues 917–928 in hPRB (Fig. 1). The location of the repressor function was further confirmed by experiments demonstrating that mutations in this region abolished the repression and converted the full-length receptor to a form which positively responds to RU486 (Fig. 2).

Two alternative hypotheses regarding the function of the C-terminal repressor domain can be envisioned: (*i*) The C-terminal region may directly silence transactivation domains of

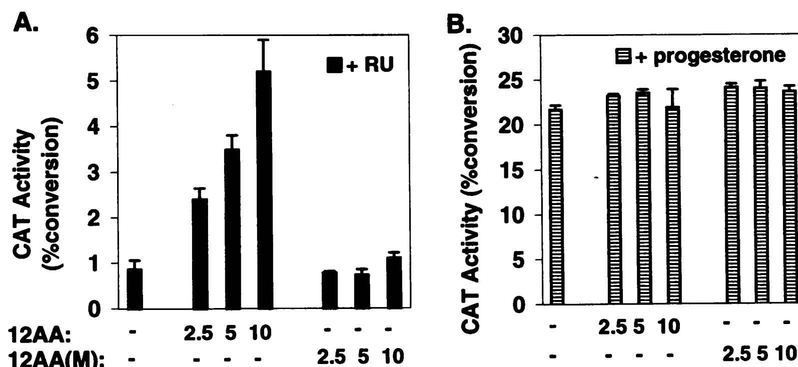

FIG. 4. Overexpression of the repressor region enhances the RU486-dependent activity of hPRB. HeLa cells were cotransfected with 0.5 μg of pRShPRB plasmids, 5 μg of PRE2-TK-CAT reporter DNA, and 0, 2.5, 5, or 10 μg of GAL4-DBD-12AA (12AA) or GAL4-DBD-12AA(m) [12AA(M)], respectively. Transfected cells were treated with RU486 (*A*) or progesterone (*B*) for 24 hr before CAT assay.

the receptor through an intramolecular mechanism (quenching); (*ii*) The C-terminal region may interact with an as yet unidentified inhibitory factor and form a complex that keeps the receptor in an inactive form (1, 10). Our data are consistent with the second model. Overexpression of the 12AA repressor region can activate the wild-type PRB in the presence of RU486 (Fig. 4). This result suggests that the repressor region located in the C terminus of the PR serves as a site for interaction with an inhibitory cofactor. Since the 12AA repressor region can repress a basal promoter activity when fused to a heterologous DNA binding domain (Fig. 3), this inhibitory cofactor most likely interacts with both the RU486-bound receptor and the basal transcriptional machinery to block initiation of transcription; the detailed mechanism is unclear at the present.

Two transcriptional corepressors, termed N-CoR and SMRT, have been shown to interact with unliganded TR and RAR. Hormone binding induces dissociation of these corepressors from receptors and leads to activation of target gene transcription (30, 31). The question is whether the corepressor required for PR is the same as those for TR and RAR. In HeLa assays, we were unable to show that overexpression of PR in the absence or presence of RU486 can release the silencing activity of TRβ by depleting corepressors. Similarly, overexpression of v-erbA, which we have shown to efficiently reverse the silencing activity of TRβ by competing for the TR corepressor (32), could not enhance the RU486-dependent activity of hPRB in HeLa cells (data not shown). Finally, it has been reported previously that the N-CoR and SMRT are unable to interact with classic steroid receptors (30, 31). Collectively, these results suggest that the corepressor for PR is likely to be distinct from those for TR and RAR, but this question remains unsettled at present.

Since unliganded steroid receptor molecules are associated with heat shock proteins and unable to bind DNA, the biologic necessity to have a corepressor to keep PR silent in the absence of progesterone has not been postulated previously. Certainly, it is possible that most of the receptors have a low level of activity due to leakage. Thus, the existence of corepressor may reduce such basal gene activity to a minimum. Alternatively, one could ask whether natural antagonists that may function like RU486 could exist *in vivo*. The existence of natural antagonists is not implausible since there are distinct binding sites for agonist and antagonist on hPR (10). If this is true, such antagonists could play an important negative regulatory role in recruiting corepressor and silencing target genes or could prevent other regulators from occupying the same DNA response element. Indeed, the hPRB inhibits basal promoter activity by 50–70% in transient transfection assays. The repression depends on both the concentration of RU486 and the expression level of hPRB (data not shown). These data, together with the active repressor function of the 12AA domain when fused to GAL4 DBD, indicate that hPRB may have intrinsic repression function when it associates with its corepressor. This repression function also may be used in cases where target genes are repressed via interactions with negative DNA response elements or transfactors bound to DNA nearby to target genes.

Taken together, our experimental data are consistent with the following model for PR function. In the RU486-induced conformation, the inhibitory domain in the C terminus remains available for interaction with an inhibitory cofactors. The association of the corepressor renders the activation domain into a nonfunctional form, and then represses target gene transcription. Upon binding to progesterone agonist, a distinct conformation in the C-terminal region is induced which decreases repressor domain interaction with its corepressor. The activation domain is now able to interact with a coactivator in the absence of corepressor and target gene activation ensues.

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