Differential Regulation of Androgen and Glucocorticoid Receptors by Retinoblastoma Protein*

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The androgen receptor (AR) plays a major role in the development and maintenance of male primary and secondary sexual characteristics. The growth promoting effects of androgens are clearly seen in prostate cancer where treatment by androgen ablation usually leads to tumor regression, followed sometime later, by growth of tumor cells that are resistant to endocrine therapy. We have found that the level of pRB in cells controls AR activity. Overexpression of pRB leads to increased transcriptional activity of the AR. This is similar to the previously reported potentiation of glucocorticoid receptor activity by pRB. In contrast, loss of pRB activity inhibits AR but not glucocorticoid receptor activity. This inhibition correlates with the unique ability of the AR to form a protein-protein complex with pRB in vitro. The site of interaction with pRB lies within the N-terminal domain of the AR and co-localizes with the region of the AR that specifies a requirement for pRB. Thus, the AR has a novel requirement for pRB raising the possibility that the growth promoting activity of AR is due to its direct interaction with pRB. Furthermore, loss of pRB activity during progression of prostate cancer may directly result in a decreased response to androgens.

The androgen and glucocorticoid receptors, members of the steroid, thyroid, and retinoic acid superfamily, consist of three structural and functional domains: a central DNA-binding domain, a C-terminal hormone-binding domain (HBD) and a N-terminal domain containing a transcription activation function (1). In the absence of hormone, these receptors are inactive due to association with a number of proteins including HSP90 and immunophilins (2). Hormone binding brings about a conformational change in the receptors that disrupts this inactive protein complex and allows the receptor to associate with DNA. The conformational change brought about by hormone binding also allows the recruitment to the HBD of transcriptional co-activators that are essential for receptor activity (reviewed in Refs. 3 and 4). Since deletion of the HBD from both the GR and AR results in a constitutively active receptor (5–10), the HBD must also have a silencing effect on the transcription activation function present in the N-terminal domain. This is exemplified by the action of antagonists that can bring about DNA binding but neither recruit coactivators nor activate transcription. The silencing effect may be due to the action of a transcriptional suppressor and/or the direct interaction of the two domains (11–13).

The GR subfamily of steroid receptors (GR, AR, progesterone receptor, and mineralocorticoid receptor) can activate the same test promoters in transfected cells (14–16). For instance, all four receptors activate the mouse mammary tumor virus promoter due to interaction with the same hormone response elements within the promoter. Presumably, the specific transcriptional responses to androgens, glucocorticoids, mineralocorticoids, and progestins seen in vivo are due, at least in part, to the differential interaction of these receptors with transcription factors and/or co-activators. We have begun a characterization of the AR and GR transcriptional pathways in an attempt to find components that are unique to one of the receptors. It has been shown previously that overexpression of pRB increases the activity of the GR probably through association of pRB with hBRM, a human homolog of Saccharomyces cerevisiae SWI2/SNF2 (17). We have found that pRB overexpression results in synergistic activation of the AR as well as the GR. In contrast to the activation of both receptors by pRB overexpression, loss of pRB activity inhibits only AR activity; there is no effect on GR activity. Since we show that AR, but not GR, can form a complex with pRB in vitro, our data suggest that the mechanism by which pRB activates the AR is different from the mechanism by which it activates the GR. Since the AR has little activity in the absence of pRB, our data also raise the possibility that the loss of androgen responsiveness seen during the progression of prostate cancer may, in some cases, be due to mutation or loss of expression of pRB.

EXPERIMENTAL PROCEDURES

Plasmids—pJL1 and pJL2 were made by removing a ClaI-EcoRV fragment containing EBNA-1 and ori-P from either pREP9 or pREP10 (Invitrogen), respectively. pJL1hAR contains a BamHI-BglII fragment containing the hAR coding region from pHAR inserted into the BamHI site of pJL1. pJL2mGR was made by inserting a BglII-XbaI fragment containing the mGR coding region from pmGR (18) into the BamHI-Xhel sites of pJL2. In both cases, the Rous sarcoma virus long terminal repeat drives expression. pHAR was made by replacing a BglII-XbaI fragment containing the mGR coding sequence in pmGR with a BglII-XbaI fragment containing the hAR coding region from pHAR. pHARAC was made by removing a Thl111I-BstBI fragment from pHAR and a stop codon was introduced immediately after Thl111I site. For in vitro translation, pGEMhARAC was made by subcloning a BglII-XbaI fragment from pHARAC into pGEM (Promega). AR 506–918 and AR 1–502 were made by removing a HincII-BglII fragment from pHhAR and a BglII-BamHI fragment of pHAR into pBluescript SK− (Stratagene), respectively. AR 1–565 and AR 1–502 were made by removing HindIII-HindIII and Kpn1-XbaI fragments from pHAR, respectively. AR L559S, AR DE563/4NQ, and DE563/4KK were made using the kit from CLONTECH with pHAR as template. Then, the mutated receptors were

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The abbreviations used are: HBD, hormone-binding domain; AR, androgen receptor; GR, glucocorticoid receptor; DHT, dihydrotestosterone; Dex, dexamethasone; CAT, chloramphenicol acetyltransferase; pRB, retinoblastoma protein; Tag, SV40 large T-antigen; HPV, human papilloma virus.
subcloned into pGEM. AR-C was described previously (19). T3A5C was made by inserting a stop codon immediately downstream of codon 44 in pSV3neo (20). pSV2-E1A12S contained the E1a coding region from pMW29-E1A12S. PHuβAcrp-1-neo-QP is a pRB expression plasmid under the control of a β-actin promoter.

Cell Transfection—Transfections using the calcium phosphate precipitation technique were performed as described previously (18). For transfections using electroporation, 4 million cells were mixed with 20–30 μg of total DNA and shocked at 300 V with 800 microfarads. CAT activities were determined as described (18). In all transfections measuring transcriptional activity, 15 μg of pMCAT or pGRE5-2 CAT and 1 μg of pCMVluc were used. Where indicated, the transfections also included 1 μg of pJL1Har or pHaHar; 0.5 μg of pJL2mgR or pmGR; 8 μg of pHuβAcrp-1-neo-QP; 8 μg of pSV3-neo, pT3A5C, or carrier DNA. Luciferase activity was used to monitor transfection efficiency. For hormone binding assays, cells were transfected with 25 μg of pHHar or 12.5 μg of pHHar plus 12.5 μg of pSV3-neo.

Hormone Binding Assays—Whole cell hormone binding assays were performed essentially as described (18). Transfected cells were incubated at 37 °C with increasing concentrations of [3H]R1881 (30 fM to 3 nM) in the presence or absence of a 1000-fold molar excess of unlabeled R1881 for 2 h. Unbound hormone was removed by washing and bound radioactivity determined by scintillation counting. The data were fit to the Michaelis-Menten equation by the method of least squares (21). The data were not normalized for transfection efficiencies.

In Vitro Binding Analysis using GST-pRB Coupled Sepharose Beads—Escherichia coli BL21 cultures expressing either GST (pGex-4T-3) or GST-RB (pGexKG-RB) were prepared as described previously except the induction factor was replaced by 2 mM IPTG. 20 mM Tris, pH 7.8 and 1% Tween 20 was added to the lysis buffer (22). AR, GR, and luciferase were synthesized using a 40-μl coupled in vitro transcription/translation system as described by the manufacturer (Promega). The reactions were carried out at 30 °C for 120 min in the presence of 2.5 μl of [35S]Met (10 μCi/μl) and with or without 5 μM of the synthetic steroid R1881 (Amersham). 10 μl of GST-Sepharose or GST-RB-Sepharose beads were resuspended in 100 μl of 2-NEt plus 0.1% Nonidet P-40, 0.1% Triton X-100, 5 μg/ml Pefabloc (Boehringer Mannheim), 5 μg/ml leupeptin. 12 μl of in vitro translation product was added and the mixture rotated at 4 °C for 90 min in the presence or absence of 2 μM hormone. The beads were washed 5 times with 2-NEt plus 0.1% Nonidet P-40 and 0.1% Triton X-100, resuspended in 40 μl of Laemmli sample buffer and proteins separated by SDS-polyacrylamide gel electrophoresis using a 7.2% acrylamide gel for the AR and GR and a 9% gel for the luciferase. For the AR deletions and mutations, the methods are the same except that the percentage of acrylamide gels is different.

RESULTS
pRB Synergizes with the AR—It has been shown previously that pRB synergizes with the GR resulting in a 4–5-fold increase in receptor activity (17). To determine whether pRB could also functionally interact with the AR, we transfected two pRB-deficient cell lines, Saos-2 and MDA-MB468, with AR and GR in the presence or absence of 8 μg of pRB (Fig. 1). In the osteosarcoma-derived cell line Saos-2 that contains an inactive, truncated form of pRB (23), AR gave a modest induction of CAT activity (Fig. 1A). Co-transfection with a pRB expression vector, however, resulted in over a 13-fold induction of CAT activity by the androgen dihydrotestosterone (DHT). As expected, pRB also increased GR activity when activated by the glucocorticoid dexamethasone (Dex). However, pRB only increased GR activity 1.5-fold due, perhaps, to the high level of hormone induction of GR seen in the absence of pRB. Similar results were obtained in the breast-cancer-derived cell line MDA-MB468 that does not produce any measurable pRB protein (24, 25). In the absence of pRB, the AR had little activity, giving no more than a 3-fold induction by hormone (Fig. 1B). Co-transfection with the pRB expression vector resulted in a 21-fold induction of CAT activity by hormone, a 7-fold increase in induction over AR alone. In a separate experiment using electroporation to transfact MDA-MB468 cells, the synergistic effect of pRB on AR activity was dose dependent (Fig. 1C). These results demonstrate that the AR has little activity in the absence of pRB and that pRB re-expression leads to greatly increased AR activity. In contrast, the GR efficiently activates transcription in the absence of pRB and is stimulated only modestly by overexpression of pRB.

AR Is Active in Cells Expressing Endogenous pRB but Activity Is Lost Upon Expression of a pRB-binding Oncogene—To test whether endogenous levels of pRB were sufficient to synergize with the AR and GR, we repeated the transfections described above using CV1 and COS-7 cells. COS-7 cells were originally derived from CV1 by transformation with a SV40 T-antigen (Tag) expression vector (26). Since Tag binds tightly to the hypophosphorylated form of pRB, COS-7 cells are functionally pRB-negative in contrast to CV1 cells that are pRB-positive (27–29). As expected, GR had high levels of inducible activity in both CV1 and COS-7 cells, whereas AR had significant activity only in CV1 cells (Fig. 2A). To ensure that the effects of Tag on AR activity were not due to effects on the AR expression vector or on the reporter pMCAT, we repeated these experiments with another hAR expression vector (pHHaAR, derived from pSV2 (21)), and the reporter plasmid pGRE5-2 CAT (30) (Fig. 2B). Again, Dex induced GR activity in both cell types while DHT only induced AR activity efficiently in CV1 cells. In the related cell line COS-1, which also expresses Tag, AR again had very low activity compared with CV1 cells (Fig. 2B). In general, we see slightly more activity in COS-1 cells compared with COS-7 cells and indeed, moderate AR activity has been reported in COS-7 cells previously (31).

To test whether Tag was responsible for the inactivity of the AR in COS-7 cells, or whether these cells had become nonpermissive for AR activity due to some other event that had occurred during derivation of the cell line, we transiently transfected CV1 cells with the Tag expression vector, pSV3neo (Fig. 2C). Wild type Tag expression did indeed inhibit the activity of the AR on pMCAT, but it had no effect on the activity of the GR on the same reporter plasmid. A truncated version of Tag that consists of just the first 44 amino acids of Tag had no effect on either AR or GR activity (Fig. 2C). Taken together, these results demonstrate that expression of SV40 Tag specifically

FIG. 1. pRB Potentiates the activity of the AR. Saos-2 (A) or MDA-MB468 (B) cells were transfected with pMCAT, pHHar, pmGR, and either 8 μg of the pRB expression vector or 8 μg of carrier DNA using the calcium phosphate precipitation method. C, MDA-MB468 cells were co-transfected with pMCAT, pHHar, and increasing amounts of pRB expression vector (50 ng, 200 ng, 1 μg, and 5 μg) by electroporation (300 V with 800 microfarads). 48 h after transfection, the cells were redistributed into 24-well plates and then induced with 50 nM DHT. Two days later, the cells were harvested and CAT activity was determined.

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inhibits the AR in CV1 and COS-7 cells and has little effect on the GR. In addition, this inhibition is independent of the specific AR expression vector, reporter plasmid, or the type of androgen used.

SV40 Tag is a multifunctional protein; it has ATPase and helicase activities, it binds to DNA, and it associates with a number of proteins including the tumor suppressors pRB and p53, the pRB related proteins p107 and p130, and the transcriptional coactivator p300 (32–34). In other small DNA tumor viruses, some of these activities are found in individual oncoproteins. For instance, the human papilloma virus (HPV) protein E7 has a pRB-binding site but no p53- or p300-binding sites, while HPV E6 just interacts with p53 (35, 36). Expression of HPV E7 inhibited AR activity in CV1 cells, albeit to a lesser extent than Tag, while HPV E6 had no effect on AR activity (Fig. 2C). Expression of adenovirus 2 E1a 12S that binds pRB and p300 (37) also inhibited AR activity (Fig. 2C); however, unlike E7 and Tag, E1a also inhibited the GR. These data suggest that Tag and E7 inhibit AR activity by binding to pRB (or possibly p107 and p130), whereas E1a inhibits GR activity by binding to p300. Since it has been shown previously that p300 is essential for the activity of a number of steroid receptors including the GR (38, 39), it follows that either association of p300 with Tag does not inhibit the activity of these receptors, or that the binding of Tag to p300 is weak and sufficient free p300 remains available for receptor activity.

Tag Inhibition of AR Activity Maps to the pRB-Binding Domain of Tag and Can Be Reversed by Overexpression of pRB—Extensive mutagenesis of Tag has revealed that the biochemical activities of this protein are essentially modular in nature. Thus, the pRB and p53-binding sites map to discrete regions of the protein as do the DNA binding, ATPase, and helicase activities (Fig. 3A). We found that the first 44 amino acids of Tag are not sufficient to inhibit AR activity (Figs. 2C and 3B). We next tested a truncated Tag construct (d1137) that expressed only the first 121 amino acids of Tag and encoded just the pRB- and p300-binding sites (40). This mutant inhibited AR activity better than the wild type Tag (Fig. 3) even though less protein was produced (wt Tag and Tag mutant 6a (see below) allow replication of the Tag expression vector, dl1137 does not; this results in an approximately 20-fold decrease in protein synthesis) (Fig. 3C). The increased inhibition of AR activity by dl1137 may be due to the loss of the p53-binding site since it is known that the binding of p53 to Tag can alter its biological activity (32–34). Since d1137 contains both pRB and p300-binding sites, we tested a construct containing mutations in the pRB-binding site. Tag mutant 6a, which is unable to bind to pRB but is expected to bind p300 (41, 42), had little inhibitory effect on the transcriptional activity of the AR (Fig. 3B). If inhibition of AR activity by Tag is due to sequestering of pRB by Tag, co-transfection of CV1 cells with both d1137 and pRB should result in relief of this inhibition. To test this, we transfected CV1 cells with AR, GR, and dl1137 with and without pRB (Fig. 3D). In the presence of pRB, GR activity increased approximately 3-fold (we routinely obtain between a 1.5- and 3-fold increase in GR activity in response to pRB) consistent with the increases we saw in Saos-2 and MDA-MB468 cells (Fig. 1) and previous reports on the effect of pRB on GR activity (17). In contrast, d1137 clearly inhibited AR but not GR activity and this inhibition was reversed by pRB co-transfection.

In the Presence of Tag, the AR Acts as a Dominant Negative Regulator of the GR—We further characterized the activity of AR in CV1 and COS-7 cells using the C-terminal truncated receptor ARΔC that lacks the hormone-binding domain. As expected, in CV1 cells this truncated receptor is constitutively active, i.e. it is active even in the absence of hormone (Fig. 4A). This reflects the inhibitory role that the C terminus of steroid receptors plays in the absence of hormone. Indeed, the activity of this receptor in CV1 cells is higher than wtAR; a phenomenon seen with other C-terminal truncated steroid receptors (7). However, ARΔC is inactive in COS-7 cells (Fig. 4A) indicating that the loss of AR activity in COS-7 cells maps to a function encoded within the N-terminal and DNA-binding domains. Since both of these domains are required for AR activity, it was not possible to use other domain deletions to further map the site. Instead, we used a chimeric receptor, AR-C, that is essentially the N-terminal domain of GR fused to the DNA and hormone-binding domains of AR (due to the use of a HindIII site at the beginning of the DNA-binding domain, the first 5 amino acids of the DNA-binding domain are of GR origin, AR CLICG to GR CLVCS, see below) (19). AR-C was active in both CV1 and COS-7 cells indicating that the inhibition seen in COS-7 cells lies within or overlaps the N-terminal domain. These data also imply that in COS-7 cells the hormone- and DNA-binding properties of the AR are intact. Indeed, direct measurement of AR hormone binding activity revealed that both CV1 and COS-7 cells were able to bind hormone (Table I). The increased hormone binding activity seen in COS-7 cells is presumably due to replication of the AR expression plasmid in these cells. Similarly, we found that DNA binding activity of the AR was present in extracts of transfected COS-7 cells as seen in electrophoretic shift mobility assays (not shown).

The data presented above suggest that in COS-7 cells the AR binds to DNA but does not activate transcription. This model predicts that in cells expressing Tag, AR will act as a dominant negative receptor on the GR since both receptors bind to the same DNA sequence. To test this, CV1 cells were transfected with GR and AR and the effect of co-transfection with Tag was
Cells were treated with 50 nM DHT and harvested 48 h later. CV1 cells were transfected with pHhAR, pHhAR above the shaded regions, the labels below refer to the boxed areas in which they reside. B, inhibitory activity of Tag mutations on the AR. CV1 cells were transfected with pMCAT, pJL1hAR, and the Tag expression vector indicated in A and treated with 50 nM DHT. Cells were harvested 2 days later. The experiment has been repeated 5 times with similar results.

A

**FIG. 3.** Inhibition of AR activity by Tag requires a pRB-binding site. A, diagrammatic representation of the functional domains of Tag (32). The labels directly above the figure refer to the shaded regions, the labels below the figure refer to the boxed areas in which they reside. B, inhibitory activity of Tag mutations on the AR. CV1 cells were transfected with pMCAT, pJL1hAR, and the Tag expression vector indicated in A and treated with 50 nM DHT. Cells were harvested 2 days later. The experiment has been repeated 5 times with similar results. C, expression of Tag mutations. CV1 cells were transfected as in B. Three days after transfection, cells were harvested. An aliquot of the cells from each transfection was used for CAT assays and the rest of the samples subjected to Western analysis as described (18) except for the use of a 15% SDS-polyacrylamide gel. The primary antibody was Pab 108 (Santa Cruz Biotechnology Inc.) which recognizes an antigenic determinant in the first 82 amino acids of Tag. The position of wild type Tag and dl1137 are shown by an asterisk and an arrow, respectively. T45C is not seen since the antibody presumably does not recognize this protein. The positions of the molecular weight markers (×10−3) are shown on the left of the gel. Lane 1, wt Tag; lane 2, 6a; lane 3, dl1137; lane 4, pT45C. * wt Tag and 6a; arrow, dl1137. D, pRB reverses the inhibition of AR by Tag. CV-1 cells were co-transfected with pMCAT, pJL1hAR, and pJL2mGR in the presence of T, 6a; 1.7 (COS-7 1.7 (COS-7 1.7 (COS-7 1.7 (COS-7)), diagrammatic representation of the functional domains of Tag (32).

**FIG. 4.** Activity of AR deletions. A, CV-1 or COS-7 cells were transfected with plhAR, plhAR53C, or AR-C together with pMCAT. Cells were treated with 50 nM DHT and harvested 48 h later. B, CV1 cells were co-transfected with pMCAT, pJL2mGR, either a wild type (pJL1hAR) or DNA binding deficient (pJL1hARC558W) AR expression vector, and a Tag expression vector as indicated. Transfected cells were transferred to 24-well plates after 48 h and treated with either 10 nM Dex alone or 10 nM Dex and increasing concentrations (1 pm, 100 pm, 10 nm, and 1 μM) of DHT as indicated, and harvested 48 h later.

| Sample              | K<sub>r</sub> (nm) | B<sub>max</sub> (fmol) |
|---------------------|-------------------|----------------------|
| CV1                 | 1.4 (p<sup>2</sup> = 0.95) | 26                   |
| CV1 + Tag           | 2.3 (p<sup>2</sup> = 0.87) | 18                   |
| COS-7               | 1.7 (p<sup>2</sup> = 0.98) | 87                   |

Table I

Hormone binding properties of the AR

tested in the presence of a constant concentration of dex and increasing levels of DHT. In the presence of Tag, DHT treatment inhibited GR activity in a dose-dependent manner (Fig. 4B). This inhibition was not seen in the absence of Tag. This dominant-negative effect of the AR on GR activity required binding of the AR to DNA since an AR point mutant that cannot bind DNA, ARC558W (43), failed to inhibit GR activity in the presence of Tag (Fig. 4B).

pRB Binds to a Region of the N-terminal Domain of AR, but Does Not Bind to GR—The most straightforward mechanism by which pRB could synergize with steroid receptors is by direct interaction. Therefore, the ability of a GST-RB fusion protein to associate with in vitro synthesized AR and GR was tested in a GST-pull down assay (Fig. 5A). The GST-RB fusion protein clearly associated with the AR but not the GR in this assay correlating with the functional requirement for pRB by the AR but not the GR. In control experiments, GST alone did not bind to AR, and GST-RB did not interact with in vitro synthesized luciferase (Fig. 5A) but did associate with the well characterized pRB-binding protein HPV E7 (44) (not shown). Interestingly, the interaction of GST-RB with the AR was independent of the presence of the synthetic androgen R1881, suggesting that the interaction does not occur through the HBD of the AR. This was consistent with the functional assays that mapped the region of the AR required for inhibition by Tag to the N-terminal domain of the receptor (Fig. 4A). To further map the site of interaction, we used a series of receptor deletions in this GST-pull down assay (Fig. 5). A fragment from amino acid 1 to 565 retained the ability to bind to pRB, whereas a fragment from 1 to 502 did not suggest that the site lies between amino acids 502 and 565. This was supported by the ability of a fragment containing amino acids 506 to 919 but not a fragment containing amino acids 623 to 919 to bind to pRB. The region 502–565 of the AR contains a putative pRB-binding site, LxCxD (amino acids 559–563), that is related to the well characterized binding site LxCxE (45). However, mutational analysis of the AR indicated that this putative pRB-binding site was not involved in pRB binding, since three different AR mutants, L559S, DE563/4NQ, and DE563/4KK retained the ability to bind to pRB in vitro. The binding site therefore lies somewhere else within the polypeptide-containing amino acids 506 to 565. Indeed, this result is not surprising since GR also has this motif, and we cannot detect binding of pRB to GR (Fig. 5A). Most importantly, these binding studies map the pRB-binding site to the same region of the AR that was implicated in the functional activity of pRB (Fig. 4A) giving strong support for a model in which pRB synergizes with the AR by direct protein-protein interaction.
ground; synthetic androgen R1881. B was translated either in the absence (−) or presence (R1881) of the GR and luciferase. AR
33% of the radioactivity added to the reactions containing AR, and 17% of the radioactivity added to reactions containing AR and luciferase. AR

Polyacrylamide gel electrophoresis. Bound proteins were removed from the beads and separated by SDS-polyacrylamide gel electrophoresis. A, the lanes marked input represent 33% of the radioactivity added to the reactions containing AR, and 17% of the radioactivity added to reactions containing GR and luciferase. AR

AR binds to pRB, and the region responsible for RB binding is located between amino acids 506 and 565. GST-RB-Sepharose beads were incubated with 35S-labeled in vitro translated receptor constructs as described under “Experimental Procedures.” Bound proteins were removed from the beads and separated by SDS-polyacrylamide gel electrophoresis. A, the lanes marked input represent 33% of the radioactivity added to the reactions containing AR, and 17% of the radioactivity added to reactions containing GR and luciferase. AR

Fig. 5. The AR binds to pRB, and the region responsible for RB binding is located between amino acids 506 and 565. GST-RB-Sepharose beads were incubated with 35S-labeled in vitro translated receptor constructs as described under “Experimental Procedures.” Bound proteins were removed from the beads and separated by SDS-polyacrylamide gel electrophoresis. A, the lanes marked input represent 33% of the radioactivity added to the reactions containing AR, and 17% of the radioactivity added to reactions containing GR and luciferase. AR

DISCUSSION

The results presented in this paper demonstrate that the androgen receptor has little transcriptional activity in cells lacking pRB activity whether due to loss of pRB expression, mutation of pRB, or to expression of a pRB-binding oncogene. This is in contrast to the GR, which is active in all pRB-deficient cells that have been tested. An AR-GR chimeric receptor mapped the region of the AR required for the functional interaction with pRB to its N-terminal domain. The same region of the AR interacted directly with pRB in a GST pull-down assay suggesting that pRB potentiates AR activity due to the formation of AR-pRB dimers. Overexpression of pRB leads to increased activity of both the AR and the GR. However, since decreased pRB levels have no effect on GR activity and since we could not demonstrate formation of a GR-pRB complex in vitro, the mechanism of action of pRB on AR and GR activity is likely to be different.

A model of the way in which pRB may synergize with the AR is shown in Fig. 6. pRB binds within the last 60 amino acids of the N-terminal domain of the AR. The binding site may extend slightly into the DNA-binding domain but mutation analysis suggests that this is unlikely (Fig. 5). We do not think that pRB interacts with transcription activation function 2, the transcription activation function in the C-terminal domain of the AR, since pRB is still required for transcriptional activity of AR truncations lacking a HBD. This implies that coactivators or suppressors that interact with the HBD only in the presence of hormone are also not involved (3). This conclusion is supported by the in vitro binding data that shows that AR and pRB can form a protein-protein complex in a hormone-independent manner. pRB may interact with proteins bound to the transcription activation function in the N-terminal domain of the receptor (9, 10, 46). Alternatively, pRB could act as a bridge between the receptor and the initiation complex. This could consist of a direct connection to the transcriptional machinery through interaction with TAFII250 (47) or to the formation of a larger complex with other pRB-binding proteins such as E2F, hBRM, or BRG1 (17).

It is generally accepted that pRB overexpression increases GR activity (17). Our data are consistent with this. However, we have found that the GR, unlike the AR, remains active in pRB-negative cells. We conclude that pRB activates the GR and AR by different mechanisms. The activation of the GR is likely due solely to the overexpression of pRB and thus may not reflect a role for pRB in GR signaling in normal cells. One possible mechanism for GR activation by overexpression of pRB is the squelching of a suppressor protein such as histone deacetylase which has been shown to interact directly with pRB (48–51).

The inhibition of AR activity by SV40 Tag mapped to its pRB-binding domain. Since Tag only binds to hypophosphorylated pRB, we conclude that AR requires the hypophosphorylated form of pRB for maximum activity. Our data predicts, therefore, that during the cell cycle the AR will be active in G0 and G1 when the hypophosphorylated form of pRB predominates but will have little activity in S, G2, or M when the hyperphosphorylated form of pRB predominates. To address this, we have examined the activity of the AR during the cell cycle in LMCAT cells. These cells express AR and GR and have been stably transfected with pMCAT. As predicted, we found that the AR induces CAT activity in G0, but not in S or G2.2 In contrast, the GR is active in G0 and S and is inactive in G2, as previously published (52).

Many tumors that arise from steroid-dependent tissues initially retain this hormone dependence, but as the disease

2 E. Martinez and M. Danielsen, unpublished data.
progresses, both hormone responsiveness and dependence are lost (53, 54). The loss of both steroid dependence and responsiveness suggests that in some cases a steroid response is incompatible with tumor growth. One possible explanation for this is that steroids promote a differentiated state that is incompatible with uncontrolled growth. Given our data, one event that is expected to bring about less constrained growth and turn off a steroid response in androgen responsive tumors, is the failure to express adequate levels of wild type pRB. Two recent studies on prostate cancer support this hypothesis. In the first, abnormally low expression of pRB was found to occur more frequently in tumors from patients treated with hormonal blockade than in untreated patients (55). In the second, a correlation was found between pRB-positive prostate tumors and increased survival of patients (56).

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