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13. ABSTRACT *(Maximum 200 words)* Progesterone has been shown to play a role in the proliferation of breast cancer cells, thus the development and use of anti-progestins, such as RU 486, may serve as a useful treatment by blocking the stimulatory effects of progesterone. Progesterone exerts its biological effects through its receptor (hPR) which belongs to a superfamily of transcription factors. All steroid receptors have been shown to be phosphoproteins and agents which regulate phosphorylation have been demonstrated to affect progesterone receptor action. It is therefore important to study the mechanism by which phosphorylation impinges on progesterone receptor function, and in particular, how it enables 8-Br-cAMP to switch the antagonist RU 486 into an agonist. In this study thus far, three hPR-B specific phosphorylation sites have been mutated to non-phosphorylatable residues an the activities and protein levels of these mutant PR examined.

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FOREWORD

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Introduction

Progesterone plays a central role in the regulation of many reproductive processes, particularly in the development of the alveolar system in mammary glands and in the establishment and maintenance of pregnancy (1-3). In addition, high doses of progesterone and its stable synthetic derivatives have been used as an anti-proliferative cancer treatment (4). Progesterone has also been shown to play a role in the proliferation of breast cancer cells (5) and, as such, the development and use of anti-progestins may serve as a useful treatment by blocking the stimulatory effects of progesterone.

Progesterone exerts its biological effects through its receptor which belongs to a superfamily of steroid/thyroid/retinoid receptors which act as transcription factors (6,7). These receptors are characterized by a highly conserved DNA-binding domain which is flanked by a poorly conserved N-terminal domain and a C-terminal ligand-binding domain. The human progesterone receptor exists as two naturally occurring A and B forms which are derived from separate messages (8,9). The two isoforms differ in size, with hPR-A being ~94,000 Da in size whereas the larger hPR-B is ~120,000 Da in size. They share the same coding sequence except that hPR-B has an additional 164 amino-acids at its N-terminal which account for its increased size. There is increasing evidence that these two isoforms are functionally distinct (10,11). Although both can be transcriptionally active, depending on the promoter context (10), hPR-B appears to be a stronger activator of gene transcription in response to hormone. hPR-A has also been shown to be a promiscuous repressor capable of repressing not only the activity of hPR-B, but also that of the estrogen, androgen, mineralocorticoid and glucocorticoid receptors (10,11).

The hormone dependent pathway of receptor activation involves binding of ligand to the receptor and dissociation of heat-shock protein complex, dimerization of the receptor and translocation to the nucleus. The receptor dimer then binds to its cognate response element in the promoter region of steroid responsive genes, and through recruitment of coactivator proteins and displacement of repressor proteins, enables transcriptional regulation of the expression of target genes.

Phosphorylation is a key regulator for transcription factors, kinases and cell cycle proteins, providing a mechanism whereby signals from other cellular pathways can be received and integrated resulting in a tightly coordinated response to stimuli. Generally, phosphorylation of transcription factors serves to modulate activity rather than acting as an on/off switch. This can occur as a result of enhancing/blocking DNA-binding activity (CREB and c-jun) (12,13), altering interaction with inhibitors (eg NFkB and c-jun) (14,15)and affecting interactions with other proteins (CREB) which leads to a subsequent decrease/increase in target gene transcription (16). There are frequently multiple phosphorylation sites on transcription factors and it is the phosphorylation of specific combinations of these sites that determines the overall effect on function.

All steroid receptors have been identified as phosphoproteins, with most showing increased phosphorylation in response to hormone (17-19). Although serine/threonine residues constitute the majority of phosphorylation sites, phosphotyrosine has also been identified in the human estrogen receptor (20,21) and retinoic acid receptor β (22). Pioneering studies in the steroid

receptor phosphorylation field identified four ser-pro motifs which are phosphorylated in the chick PR, two of which are constitutively phosphorylated and two of which are highly hormone inducible (17,23). More recently, phosphorylation of the human PR in T47D cells has been shown to increase in response to hormone (24). Subsequently, seven in vivo phosphorylation sites have been identified for the human PR, Ser^{81}, Ser^{102},Ser^{162},Ser^{190},Ser^{294},Ser^{345} and Ser^{400}, with Ser^{102}, Ser^{294} and Ser^{345} being hormone inducible and three of these sites being specific to hPR-B: Ser^{81}, Ser^{102} and Ser^{162} (25,26). We expect the remaining one or two sites to be confirmed within the next year. Thus far in our functional analysis we have found that mutation of Ser^{81} to a non-phosphorylatable alanine results in a decrease in transactivation compared to wild-type hPR-B.

There is increasing evidence to suggest that like estrogen, progesterone may induce strong proliferation of breast epithelium (5). Indeed, in mice lacking PR, terminal end bud proliferation and differentiation is severely compromised (27). RU 486 has been shown in vivo to act mainly as a classical progesterone antagonist and such antagonists have been shown to inhibit the development of mammary gland buds (28), suggesting a clinical role for RU 486 in the treatment of breast cancer. In addition to being activated in a hormone-dependent manner, the chick PR can also activate target genes in response to kinase modulators such as 8-Br-cAMP and okadaic acid, a phenomenon described as ligand-independent activation of the receptor (29). Although the human PR is unable to undergo ligand-independent activation as such, response to hormone is significantly augmented in the presence of these agents (24). Potentially more important is the observation that in the presence of 8-Br-cAMP, RU 486 switches from an antagonist to an agonist (30). The ramifications of this are immediately apparent, especially since deregulation of

cancer cell growth can be due to over-expression of oncogenes coding for kinases which subsequently alter the phosphorylation status of proteins inside the cell. It is clearly necessary to understand the mechanism by which this switch can occur.

My hypothesis is that the B-specific region of hPR confers unique functions to the hPR-B isoform via at least 3 unique phosphorylation sites and interaction with another protein(s), resulting in the separate transactivation function (AF3) assigned to this region, the increased transcriptional activity of hPR-B compared to hPR-A in response to hormone, and the ability of hPR-B to mediate a functional antagonist/agonist switch for RU 486.

The aim of this project is to functionally characterize the hPR-B-specific phosphorylation sites Ser^{81}, Ser^{102}, and Ser^{162}, with a view to examining the role of phosphorylation in receptor-mediated transactivation and interaction with other proteins, and the antagonist/agonist RU 486 switch.

BODY

Methods:

Cell culture and transient transfection

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Gibco BRL) containing 10% fetal bovine serum (FBS). Cells were plated in DMEM containing 5% charcoal stripped serum at a confluency of 50-60%. On the day of transfection the medium was replaced with fresh DMEM (no serum). Adenovirus mediated transfection was carried out according to the protocol in Allgood et al (31). Briefly, the desired amount of receptor and reporter DNA was mixed with adenovirus at a multiplicity of infection of 250:1. Following incubation at room temperature (RT) for 30 min, polylysine was added and a further incubation at RT for 30 min was carried out. The cocktail was then added to each well and incubated at 37°C/5%CO2 for 2 hours (h). An equal volume of DMEM containing 10% charcoal stripped serum was then added to each well to bring the final serum concentration to 5%. 24h post-transfection, cells were treated as indicated and the following day the cells were harvested in 0.4M sodium chloride/Tris/EDTA/monothioglycerol and after undergoing three cycles of freeze/thawing were collected by centrifugation and the protein concentration determined by Bradford assay (Biorad). Cell extracts containing equivalent amounts of protein were then assayed for chloramphenicol acetyl transferase activity using a liquid assay.

For the studies with the Schule series of reporters, cells were transfected with Lipofectamine according to the manufacturer's directions (Life Technologies Gibco BRL) and assayed as described above.

Whole cell hormone binding assay

Cells were transfected using the adenovirus method. 48h post-transfection medium was removed from the cells and replaced with DMEM containing decreasing concentrations of [^{3}H]R5020 (Amersham) (1-0.05nM) and incubated for 2h at 37°C/5%CO_2. The medium was then aspirated and the cells washed three times with ice cold PBS. 1ml ethanol was added and left at RT for 15min. 800ul of each sample was added to 8ml of scintillation fluid and released [^{3}H]R5020 counted in a liquid scintillation counter (Beckman). The amount of bound radioactivity was determined by subtracting mock transfected wells from PR transfected wells. The amount of free radioactivity was determined by subtracting the amount of bound radioactivity from the original amount of radioactivity added as determined by counting the radioactive medium before addition to the wells.

Construction of mutants

Mutants were constructed using the Clontech transformer mutagenesis kit. The selection oligonucleotide is: TTCACACCGCATATGGTGCACTCT. The mutant oligonucleotides are as follows: GCAGTCGCTGGCTGACGTGGAGG (for Ala^{81}); GACCAGCAGTCGCTGGCGG ACGTGGAGGGCGCA (for Glu^{81}); GGCAGCAGTTCTGCTCCCCCAGAAAAGG (for Ala^{102}); and ACCCAGCGGGTGTTGGCCCCGCTCATGAGCCGG (Ala^{162}).

Western analysis

Equivalent amounts of protein are electrophoresed on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (BA85; Schleicher and Schuell). The #1294 anti-PR antibody was used according to Dean Edwards' protocol. A secondary rabbit anti-mouse antibody (Zymed)

followed by a horseradish peroxidase conjugated donkey anti-rabbit antibody (Amersham) were used prior to detection of the signal using enhanced chemiluminescence (Amersham).

Results and Discussion:

Mutation of Ser^{81} to alanine results in a decrease in transactivation compared to wild type PR

Cells were transfected with wild type and mutant PR, and CAT activity assayed. Fig. 1 shows that compared to wild type hPR-B, the Ala^{81} mutant has decreased transcriptional activity in CV-1 cells using a simple GRE_2E1b-CAT promoter. A number of cell lines were subsequently examined to determine if different cellular contexts would affect transcriptional outcome. Fig. 2 shows that in Hela cells, transcriptional activity of the Ala^{81} mutant is still lower than that of wild type hPR-B. Cell lines that were tested with similar results include COS and MCF-10. Since the decrease in activation could be due to a decrease in hormone binding activity, or a decrease in the overall amount of protein expressed in the cell, rather than a decrease in the intrinsic activation capacity per molecule of receptor, it was necessary to examine these possibilities.

Mutation of Ser^{81} to Ala^{81} does not cause phosphorylation at alternate sites.

In order to confirm that mutating the Ser^{81} phosphorylation site did not result in ectopic phosphorylation elsewhere on the receptor and that this mutation did indeed abolish phosphorylation at this site, cells were labeled *in vivo* with [^{32}P]-orthophosphate and phosphopeptide maps obtained show the absence of phosphopeptide containing Ser^{81}, and the

absence of any alternate phosphopeptides compared to wild type receptor. This work was carried out in our collaborator, Dean Edwards' laboratory.

Mutation of Ser^{81} to Glu^{81} does not radically alter receptor phenotype.

The Glu^{81} mutant was constructed as described in methods and analyzed alongside the wild type hPR-B and Ala^{81} mutant. As is seen in Fig. 3, mutation of Ser^{81} to Glu does not drastically alter PR activity and brings it a little closer to that of wild type receptor as might be expected if the constant negative charge provided by the glutamic acid residue is able to mimic the negative charge provided by the phosphate group.

Wild type hPR-B and Ala^{81} mutant have the same hormone binding affinity.

COS cells were transfected with either wild type hPR-B or Ala^{81} mutant and whole cell binding assays performed with [^{3}H]-R5020 to determine hormone binding affinity by Scatchard analysis. Experiments performed 5 times, (Fig. 4) showed that both hPR-B and the Ala^{81} mutant bind R5020 with very similar affinity, Kd = 0.5nM. Of note is the shifting of the Ala^{81} mutant slope to the left indicating a decrease in the number of hormone binding sites, possibly reflecting lower expression of the Ala^{81} mutant protein.

Western analysis of wild type hPR-B and Ala^{81} mutant PR

Given the results of the hormone binding assay which showed a shift to the left for the Ala^{81} mutant PR indicating a decrease in the number of hormone binding sites, and that the mutant phenotype shows a decrease in transactivation, I wished to determine the expression levels of wild type hPR-B and the Ala^{81} mutant in cells transfected with close to physiological amounts of receptor. These studies proved technically challenging since the antibodies for PR are notoriously low affinity. When I began these studies, I could only detect PR protein in cells transfected with saturating amounts of receptor DNA, and at these levels of DNA there were no apparent differences in receptor expression level. However, it was important to me to be able to analyze receptor protein when the DNA in the transfection was in the same linear range used for the CAT assays where I was able to see a difference in transactivation between wild type hPR-B and the mutant; preferably, out of the same cell extract.

Recently, I have been able to do this using a new antibody developed in our collaborator, Dean Edwards', laboratory. Fig. 5 shows that in HeLa cells where transcriptional activity of Ala^{81} mutant is lower than that of wild type hPR-B, the protein levels appear to mirror the transcriptional activity suggesting that this drop in activity may be due to a decrease in protein expression or a more rapid turnover/decreased stability of the Ala^{81} mutant PR. These results are preliminary, however, and will be repeated in order to establish if this is indeed true. Other ways of analyzing the same question include ^{35}S-methionine labeling of cells to steady-state and determining turnover for wild type versus mutant PR. In addition, examining protein levels after

blocking protein synthesis with cycloheximide will address the same question. These studies are in progress.

Analysis of hPR-B and Ala81 transcriptional activity on a complex promoter.

Recently, it is becoming apparent that receptor-mediated transcription of target genes involves recruitment of proteins such as coactivators and corepressors that act as a bridge between the receptor and the transcriptional machinery of the cell. Phosphorylation of steroid receptors may then affect the interactions with these and other possible transcription cofactors that might be needed for target gene regulation. Such an effect is more likely to be detected on complex promoters which contain binding sites for other transcription factors rather than the simple response elements contained within $GRE_2E1bCAT$. The transcriptional activity of hPR-B wild type versus the Ala^{81} mutant PR was therefore examined using a mouse mammary tumor virus promoter linked to a CAT reporter (MMTV-CAT). Fig. 6 shows that in both Hela and CV-1 cells the Ala^{81} mutant PR still appears to show reduced activity although it is somewhat less than the reduction observed with the $GRE_2E1bCAT$. Although no dramatic change in the phenotype of the phosphorylation site mutant PR was observed in this case, it remains important to investigate a variety of complex promoters which may be able to define the role of specific phosphorylation sites in interacting with particular transcription factors.

Analysis of Ala^{102} mutant PR transcriptional activity

The Ala^{102} mutant was constructed as described in Methods and analyzed by transfection and CAT assay. Using a $GRE_2E1bCAT$ reporter in HeLa cells, the activity of the Ala^{102} mutant does not appear to be compromised compared to wild type hPR-B as seen in Fig. 7. Since it was necessary to determine protein expression levels before any conclusions could be drawn, Western analysis was performed on the samples.

Western analysis of Ala^{102} mutant.

When analyzing protein levels for the Ala^{102} mutant it became apparent that mutating Ser^{102} to alanine eliminated the slower migrating upper band observed with wild type hPR-B using Western analysis (Fig. 8). This suggests a distinct conformational change in the receptor in the absence of phosphorylated Ser^{102}. The levels of receptor protein, however, again seem to reflect that amount of transactivation relative to wild type hPR-B suggesting that under these conditions phosphorylation of this site may not affect transcriptional activity.

Hormone binding analysis of the Ala^{102} mutant

In order to confirm that the hormone binding affinity of the Ala^{102} mutant was not altered, hormone binding assays were carried out. Fig. 9 shows a whole cell hormone binding assay for Ala^{102} and wild type hPR-B. Scatchard analysis shows the affinities of both wild type and

mutant PR for R5020 are virtually indistinguishable with a Kd of 0.16 nM for wild type hPR-B and 0.20 nM for the Ala^{102} mutant.

Analysis of the Ala^{162} mutant

The Ala^{162} mutant was constructed as described in Methods. Cells were transfected with wild type hPR-B and Ala^{162} mutant to examine transcriptional activity. As is shown in Fig. 10, the Ala^{162} mutant PR does not show a dramatic difference in transcriptional activity as compared with wild type hPR-B.

Western and hormone binding analysis of the Ala^{162} mutant.

Western analysis of the Ala^{162} mutant indicates no apparent differences between mutant and wild type hPR-B as shown in Fig. 11. Whole cell hormone binding assay also indicated no significant difference in affinity of the wild type and mutant receptors for R5020 (Kd = 0.16 nM for wild type hPR-B and 0.157 nM for Ala^{162} mutant (Fig. 12).

Analysis of antagonist/agonist switch.

All three of the B-specific mutants were transfected into CV-1 cells and analyzed for their ability to respond to RU 486 as an agonist in the presence of 8-Br-cAMP. In addition, a double mutant ($Ala^{102,162}$) and a triple mutant ($Ala^{81,102,162}$) were also tested. As shown in Fig. 13, all of the

hPR-B mutants examined appear to retain the low amount of "switch" activity observed in the presence of RU 486 and 8-Br-cAMP.

Analysis of Schule series of promoters.

As mentioned earlier, since the activity of any given phosphorylation site mutant PR might depend on with what co-transcription factors it interacts, I obtained a series of promoters from Schule (32) which all contain a single GRE in addition to another transcription factor binding site. A given phosphorylation site mutant PR may bind to a particular promoter and induce CAT activity differentially depending on whether phosphorylation of that site is necessary for activation in that particular promoter context. I initially attempted to characterize these promoters using our highly efficient adenovirus-mediated DNA transfer method for transfection. Perhaps because of its efficiency however, I was able to obtain only a low signal to noise ratio as the background was consistently so high. Hence, I have now moved to the less effective lipofectamine method of transfection which allows me to obtain a better signal to noise ratio at low receptor levels. Fig. 14 shows preliminary evidence with these reporters under conditions that I believe will be useful for determining any differences between the wild type and phosphorylation site mutant PR.

Conclusions:

Analysis of the Ala^{81} phosphorylation site mutant has revealed that obliterating phosphorylation of serine 81 results in a decrease in transactivation of a target reporter gene by this receptor.

Whether this observed decrease is intrinsic to the mutant receptor and is due to reduced activity per molecule of the receptor, or whether the absence of phosphorylation compromises the stability of the receptor leading to a consequent decrease in activation of the reporter gene remains to be clarified. Since this is an important question I am undertaking studies to determine the mechanism by which phosphorylation of Ser^{81} affects receptor function. Scatchard analysis indicates that it is not due to a decrease in affinity of the mutant PR for R5020. The preliminary Western analysis and hormone binding assay data, however, suggest a decrease in the amount of mutant PR protein compared to wild type. I will therefore examine protein stability of the two receptors using cycloheximide to block protein synthesis and measuring the turnover of receptor proteins from 0-72h using Western analysis.

Preliminary analysis of the other two B-specific phosphorylation sites, Ser^{102} and Ser^{162}, shows no dramatic phenotypes under the conditions tested thus far using a simple promoter. However, now that I have established conditions for examining the Schule series of reporters which contain a single PRE binding site in addition to a binding site for another transcription factor, I will be able to examine the potential importance of phosphorylation in PR-mediated transcription from complex promoters. Interestingly, mutating Ser^{102} to alanine results in the disappearance of the upper migrating band generally detected on wild type hPR-B by Western analysis. This suggests that phosphorylation of Ser^{102} may change the conformation of the receptor such that it migrates slower through the gel and absence of this phosphorylation results in the receptor no longer migrating as a triplet of protein species.

The studies undertaken thus far have been under conditions where charcoal stripped serum has been used in the medium. This is generally accepted as a way of reducing non-specific background activation resulting from contaminating steroids in full serum. Unfortunately, heat inactivation and stripping of the serum also eliminate important growth factors that are necessary for regular cell growth and cell signaling. This becomes an issue when studying phosphorylation which requires many intricate signaling pathways to be operating in the cell. It will clearly be useful to examine the activities of the phosphorylation site mutant PR constructs under conditions which may give a truer indication of what is actually occurring. For example, we know that casein kinase II and cdk2 phosphorylate Ser^{81} and Ser^{162}, respectively, of the hPR-B-specific sites. By over-expressing these kinases in cells we may be able to see an enhancement of wild type hPR-B mediated hormone-dependent activity not detectable with a particular phosphorylation site mutant PR, indicating a role for that phosphorylation site which would otherwise not be observable under the conditions currently being used.

All the B-specific phosphorylation site mutants appear not to be compromised in their ability to confer agonist activity on RU 486 in the presence of 8-Br-cAMP on a simple promoter. Recently, a phospho-specific antibody which recognizes phosphorylated Ser^{294} on PR has been developed by our collaborator, Dean Edwards. Interestingly, this antibody appears to recognize hPR-B much more strongly than hPR-A in T47D breast cancer cells. This suggests that in vivo, this site is almost exclusively phosphorylated on the B-form of the receptor, making this another target for exploration with regards to the antagonist/agonist switch since the "switch" activity is specific to the hPR-B form of the receptor.

The work carried out to date on this project correlates very well with the statement of work in my original proposal.

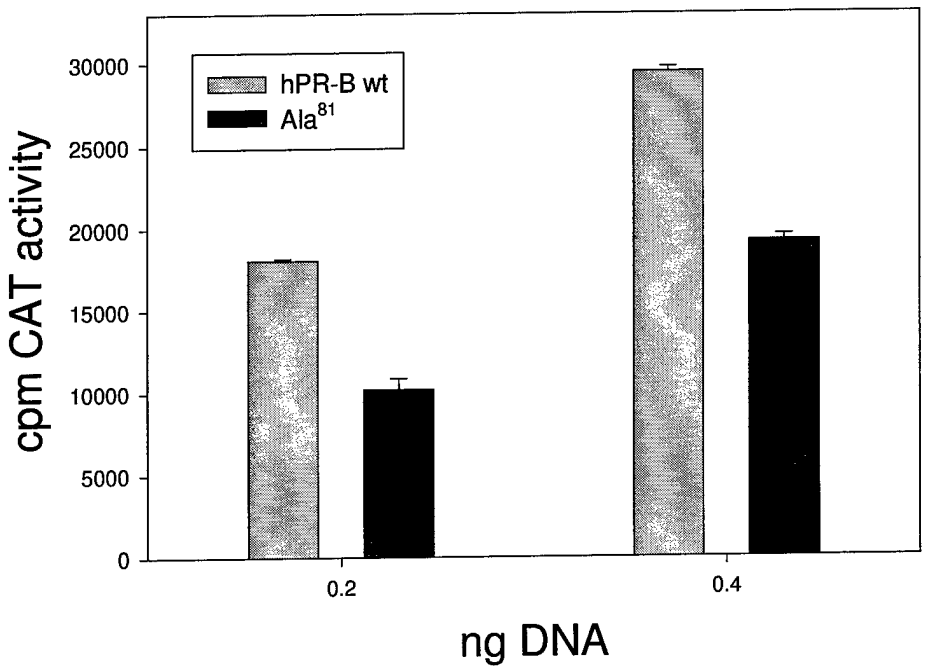

Figure 1: Comparison of hPR-B wild type and Ala^{81} PR using a $GRE_2E1bCAT$ reporter in CV-1 cells. Cells were treated with 10^{-8}M R5020.

Comparison of hPR-B and Ala^{81} PR activity in HeLa cells

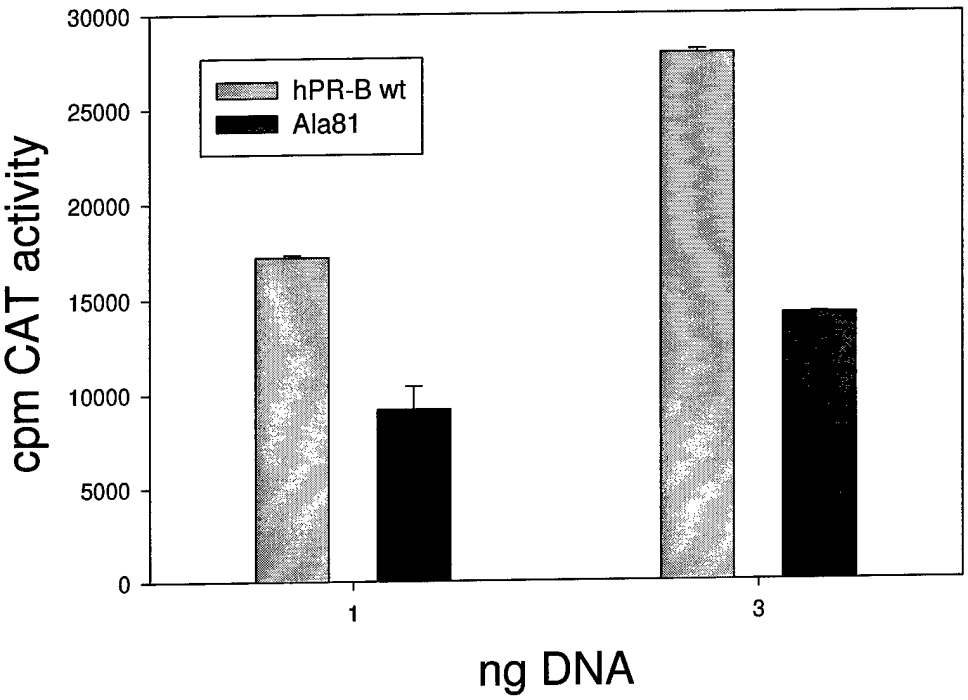

Figure 2: Comparison of hPR-B and Ala^{81} PR using a GRE$_2$E1bCAT reporter in HeLa cells. Cells were treated with 10^{-8}M R5020.

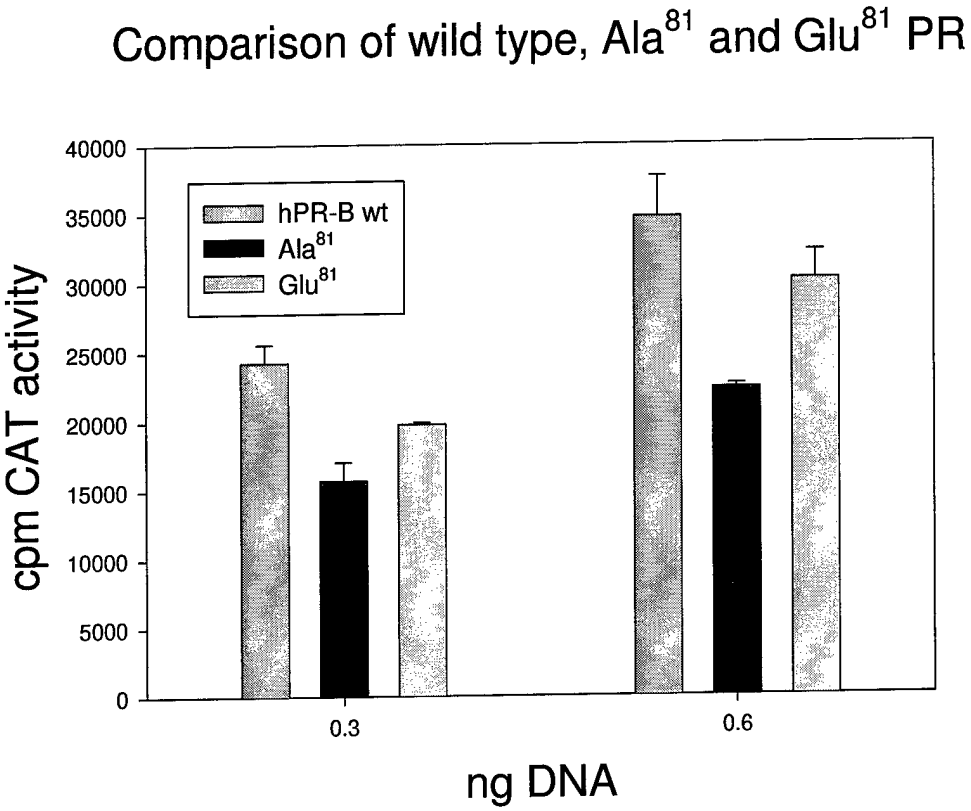

Figure 3: Comparison of hPR-B wild type, Ala^{81} and Glu^{81}mutant PR with $GRE_2E1bCAT$ reporter in COS cells. Cells were treated with 10^{-8}M R5020.

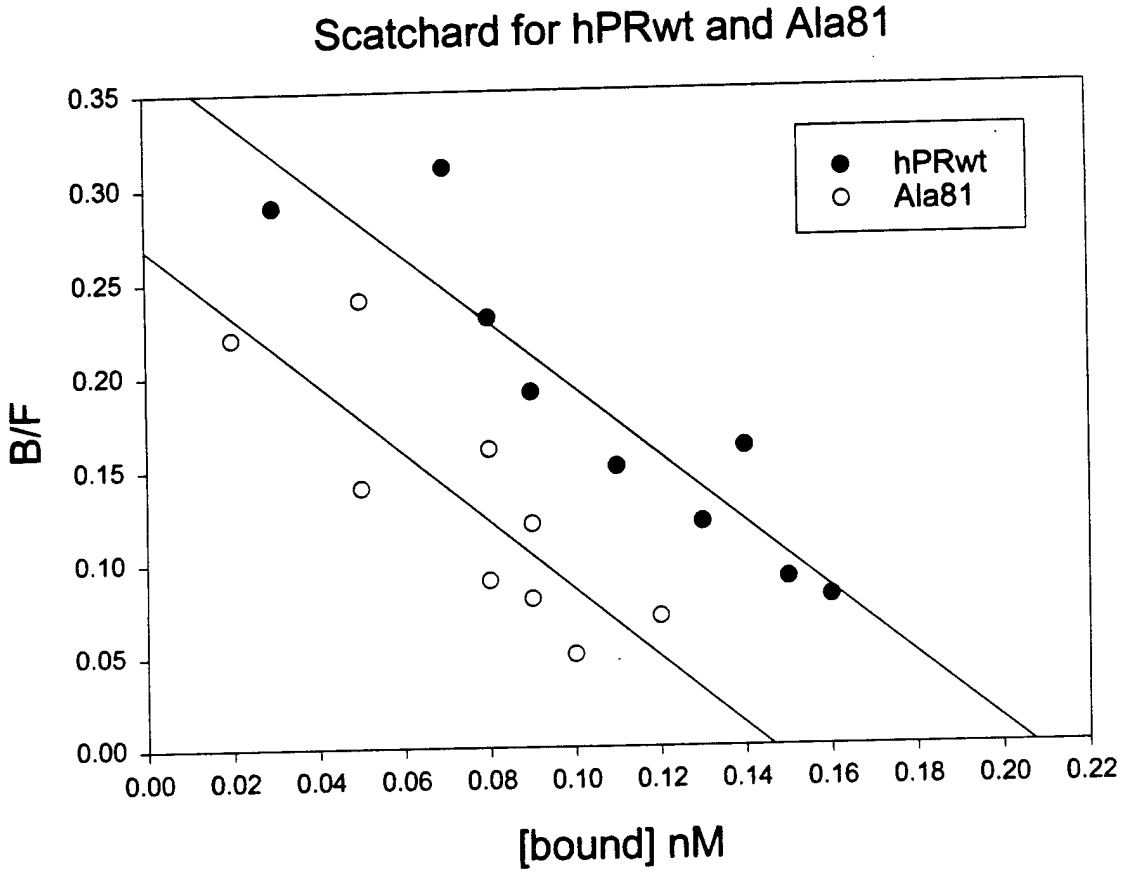

Figure 4: Scatchard analysis of hPR-B wild type and Ala^{81} mutant PR. Cells were transfected with either hPR-B wild type, Ala^{81} or empty pLEM vector and whole cell binding assays were carried out as described in Methods.

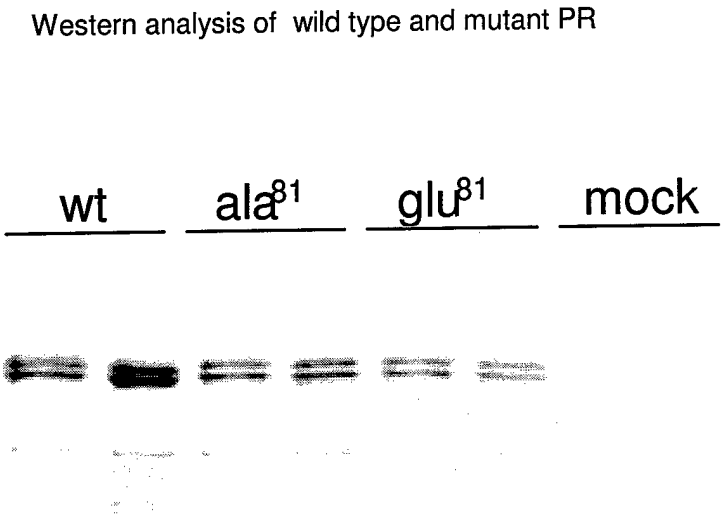

Figure 5. Wild type and mutant hPR-B (3ng) were transfected into HeLa cells. Mock transfected cells received pLEM empty vector. 30ug cell extract was separated by SDS-PAGE and probed with the #1294 antibody.

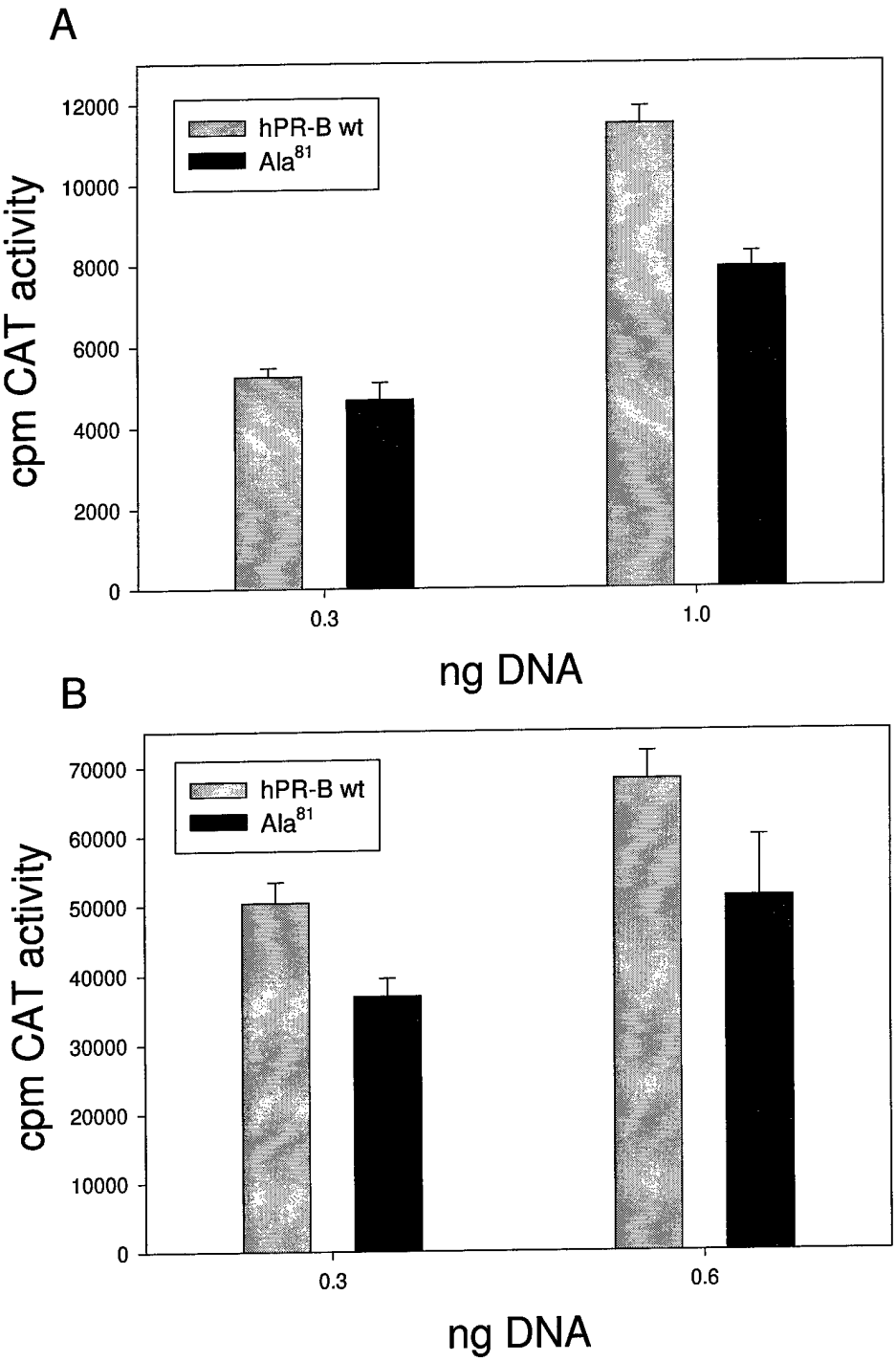

Figure 6: Comparison of hPR-B and Ala^{81} PR with MMTV-CAT reporter in A) HeLa cells and B) CV-1 cells. Cells were treated with 10^{-8}M R5020

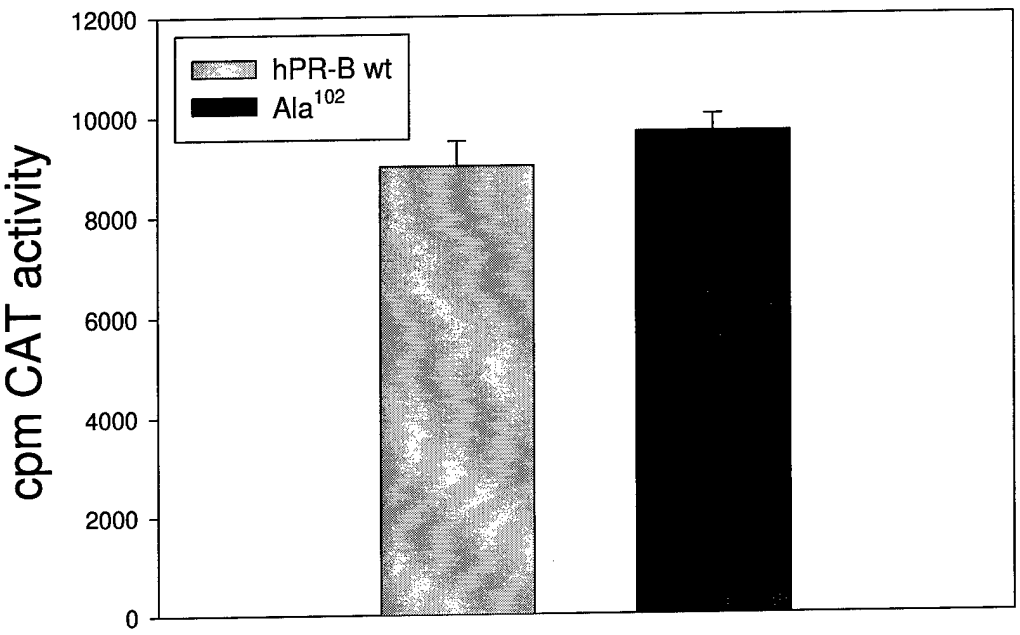

Figure 7: Comparison of 0.5 ng hPR-B wild type and Ala^{102} PR with $GRE_2E1bCAT$ reporter in CV-1 cells.

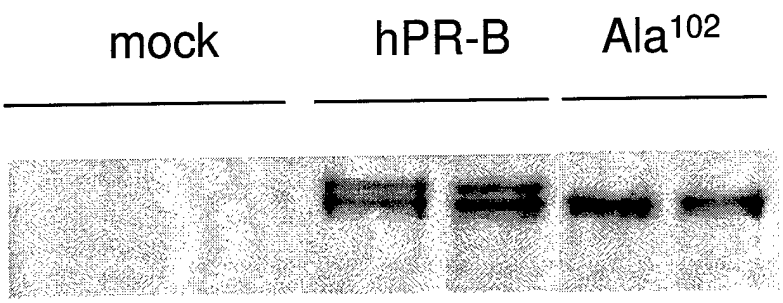

Figure 8: Western analysis of hPR-B wild type and Ala^{102} PR in CV-1 cells

Scatchard analysis of wt and A^{102} PR

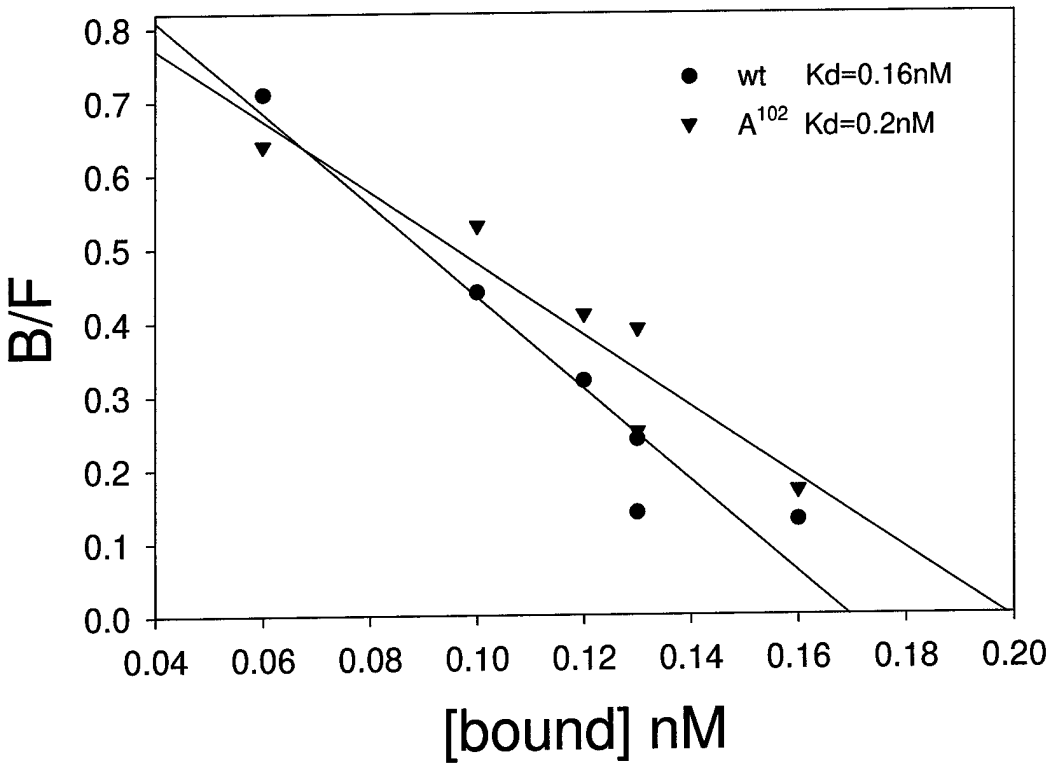

Figure 9: Scatchard analysis of wild type hPR-B and the Ala^{102} mutant. Whole cell binding assays were carried out as described in Methods.

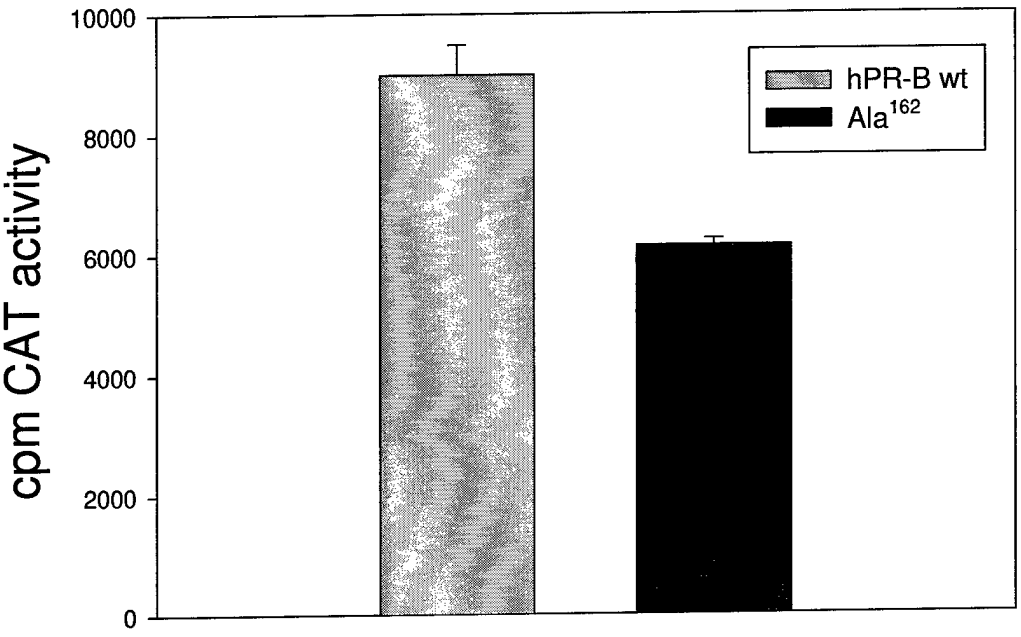

Figure 10: Comparison of 0.5ng hPR-B wild type and Ala^{162} mutant PR with $GRE_2E1bCAT$ reporter in CV-1 cells.

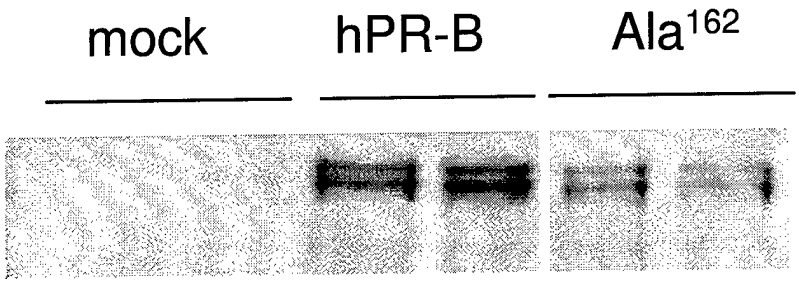

Figure 11: Western analysis of hPR-B wild type and Ala^{162} PR in CV-1 cells

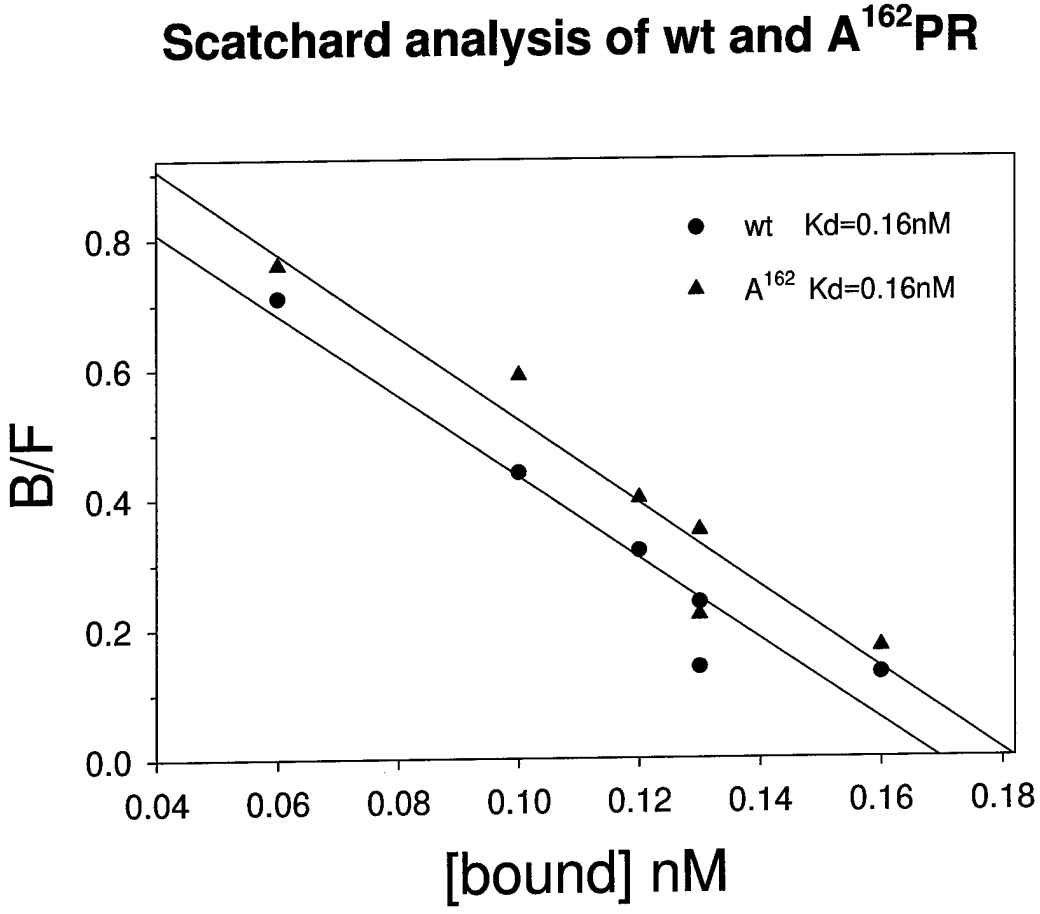

Figure12: Scatchard analysis of wild type and A^{162} mutant PR.
Whole cell binding assays were carried out as described in Methods.

hPR-B specific phosphorylation site mutants exhibit antagonist/agonist switch

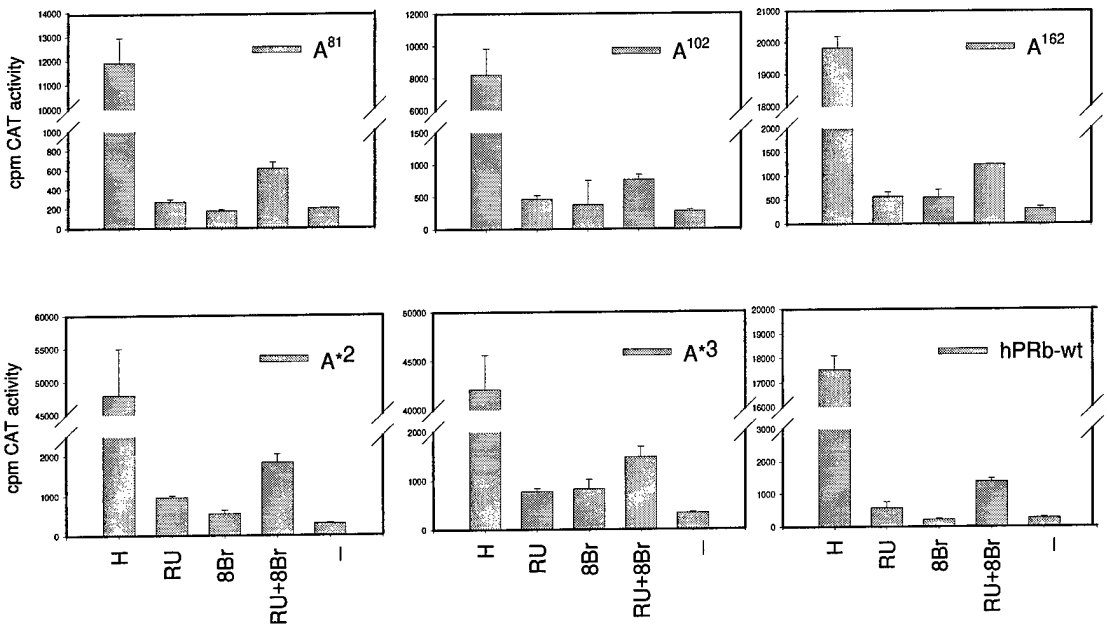

Figure 13: CV-1 cells were transfected with $GRE_2E1bCAT$ reporter and PR. Treatments were as follows: 10^{-8}M R5020 (H), 10^{-8}M RU486 (RU), 1mM 8-Bromo-cAMP (8Br) or no treatment (-).

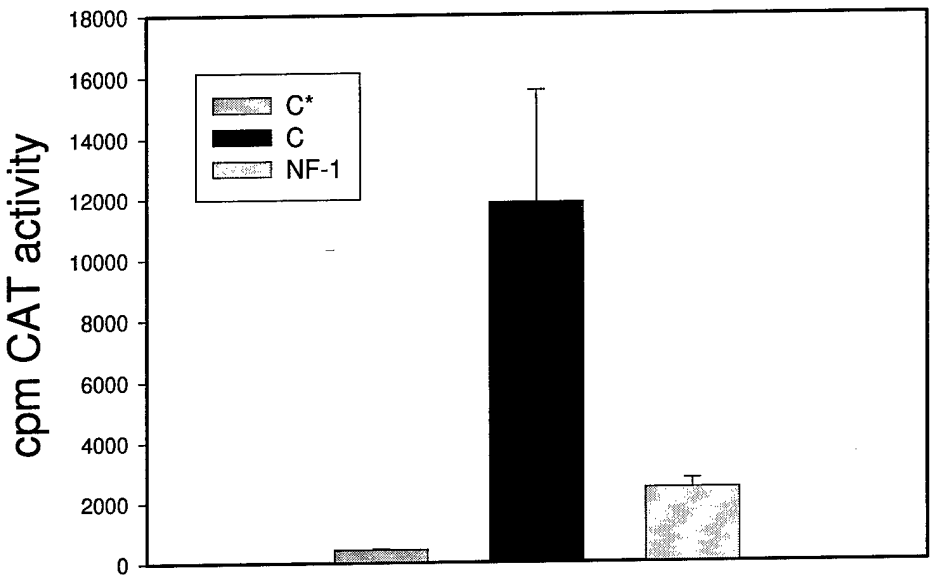

Figure 14: Comparison of CAT activity induced with three reporters (0.4 ug) from the Schule series with hPR-B (2.5ng) in HeLa cells. Background obtained with receptor, reporter and no hormone was subtracted from the hormone treated samples.

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