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Synergistic Activation of Yeast-expressed Rat Androgen Receptor by Modulators of Protein Kinase-A

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We have employed a yeast (*Saccharomyces cerevisiae*) based rat androgen receptor expression system to examine the cross-talk between different signalling pathways. We report here the synergistic modulation of androgen regulated transcriptional activation of β-galactosidase reporter activity by the activators of protein kinase-A, like forskolin and 8-bromo-cyclic AMP. A similar ligand-dependent enhancement of reporter activity compared to a DHT treated control has been noticed with okadaic acid, which is a potent inhibitor of protein phosphatase. The activation could be blocked by protein kinase-A/C inhibitor, H7. Forskolin treatment neither altered levels of receptor mRNA nor [^{3}H]R1881 binding to the receptor. Although it promotes binding of receptor to an androgen response element, forskolin was unable to activate subsequent interaction with the transcription machinery in the absence of androgen. Additionally, the synergistic actions of these activators were independent of the degree of androgen response element occupancy. Anti-androgens, cyproterone acetate and flutamide, which failed to exhibit antagonistic behaviour with yeast expressed receptor, were able to antagonize only the forskolin mediated augmentation of reporter activity. Finally, analyses of mutants established the role of DNA and steroid binding domains of receptor for this synergism.

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Keywords: androgen receptor; PKA modulators; transcriptional activation; antagonist; phosphorylation

Introduction

The involvement of androgens in homeostasis and maintenance of normal male reproductive functions, including sexually dimorphic characteristics in non-genital tissues, is well known (Mooradian *et al*., 1987). Natural and synthetic androgens are also used in different clinical situations, and at present the possibility of using them to combat wasting in patients with AIDS as well as in other chronic diseases including cancer is being investigated (Holzman, 1996). Androgen action in target cells is mediated by high affinity intracellular receptors which belong to the steroid-thyroid hormone superfamily of ligand-modulated DNA-binding proteins that act directly in altering cellular gene expression (Tsai & O'Malley, 1994). These have become a major focus of biological and clinical investigation, especially with respect to the molecular mechanism of the receptor-mediated signal transduction pathway. To perform its extremely diverse functions, androgen receptor (AR) links intracellular signals directly to transcriptional responses. The protein coding region of AR comprises eight exons designated A-H, separated by seven intervening introns. Three major functional domains, the transcription regulating amino-terminal domain, the central DNA binding domain and the carboxy-terminal hormone binding domain have been identified. Deletion analyses and the use of chimeric constructs have identified activation regions of transcription factors, TAF-1 and TAF-2, which are located in amino-terminal and hormone binding domains, respectively. Among them, transcription activation of the TAF-2 region is ligand inducible. In fact TAF-2 of hormone binding domain in conjugation with TAF-1 of amino-terminal domain acts synergistically to activate transcription in a promoter and cell-specific manner.

Abbreviations used: AR, androgen receptor; ARE, androgen response element; 8-Br-cAMP, 8-bromo-cyclic AMP; Fsk, forskolin; PKA, protein kinase-A; CA, cyproterone acetate; R1881, methyltienolone; DHT, dihydrotestosterone; EMSA, electrophoretic mobility shift assay.

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Following ligand induction, AR regulates transcription by binding as a homodimer to specific upstream DNA sequences in the target genes (Tsai & O'Malley, 1994), known as the androgen response element (ARE). Thus, the regulation of a particular gene depends on the interaction of these DNA-bound receptors with the transcriptional machinery in a manner that can be modulated by co-activators or repressors (Mangelsdorf & Evans, 1995).

Several recent reports have indicated that different signal transduction pathways are coupled (Brann *et al*., 1995; Chin & Yen, 1996; Martinez-Lacaci & Dickson, 1996). Such coupling can combinatorially increase possible regulatory strategies, thereby providing a mechanism for cell and tissue-specific responses without the requirement for entirely new signalling pathways. Steroid hormone receptors including the rat AR are phosphoproteins and there is compelling evidence to suggest that the activity of several such phosphoproteins could be regulated by reversible phosphorylation (Moudgil, 1990; Orti *et al*., 1992). However, the exact role of phosphorylation on receptor function seems to be elusive. The agents, like 8-bromo-cyclic AMP (8-Br-cAMP), forskolin (Fsk), okadaic acid, vanadate, growth factors, dopamine, etc. which modulate kinase-phosphatase pathways, have been shown to augment the transcriptional activation of genes regulated by different steroid hormone receptors. Such cross-talks between the signalling pathways could either be ligand dependent or independent. For example, chicken progesterone (Denner *et al*., 1990; Power *et al*., 1992), human androgen (Nazareth & Weigel, 1996) and estrogen (Smith *et al*., 1993) receptors have been reported to be activated by these compounds in a steroid independent manner. On the other hand, ligand dependency in a similar interaction has been reported in experiments with human progesterone (Beck *et al*., 1992), glucocorticoid (Moyer *et al*., 1993; Nordeen *et al*., 1994) and rat androgen (Ikonen *et al*., 1994) receptors. Apart from such controversy regarding ligand dependency, these interactions seem to depend on the type of mammalian cells being used in the experiments. As for example, following expression of human AR in CHO cells modulators of protein kinase-A (PKA) were unable to exhibit any influence on a receptor-mediated signal transduction pathway (de Ruiter *et al*., 1995). Moreover, the involvement of receptor-associated proteins and/or other putative non-receptor participants, which influence steroid receptor mediated signalling, have not been envisaged as yet. Despite all these discrepancies, there is no doubt about the cross-talk between disparate signalling pathways. This aspect seems to have exciting biological implications, since it may explain the contribution of different factors in genesis and progression of several diseases and thus demands further elucidation.

It would be rather difficult, if not impossible, to unravel the underlying mechanism of such complex interactions between different signalling pathways (Beck *et al*., 1992) exploiting different mammalian cell lines. We therefore sought to establish a model system utilizing an experimentally simple and genetically manipulable organism, like yeast (*Saccharomyces cerevisiae*), which could be helpful in elucidating this aspect. It is well known that many of the biological processes are evolutionary conserved among species as divergent as yeast and mammals (Botstein & Fink, 1988; Gill & Tjian, 1991; Nathan & Lindquist, 1995). Therefore, instead of mammalian cells, analysis of such a complex process in a heterologous setting could be profitable for a better understanding of such cross-talks, especially if one can take advantage of the power of yeast genetics (Struhl, 1983; Botstein & Fink, 1988). In fact, interaction of different co-activators in the mammalian steroid hormone mediated signalling pathway has already been identified by employing yeast systems (Hong *et al*., 1996).

We have recently reported a controlled expression system with rat AR in yeast, *S. cerevisiae* strain YCR1, utilizing *Escherichia coli lacZ* as a reporter. Upon transformation of rat AR, β-galactosidase activity in YCR1 was found to be inducible by androgens, like DHT (dihydrotestosterone), testosterone and R1881 (Rana *et al*., 1998). To investigate the coupling of phosphorylation dependent cell signalling pathways to androgen action, we employed this heterologous system and examined the effect of the modulators of PKA-mediated cell signalling pathway in influencing AR-mediated reporter gene transactivation. As has been observed with mammalian cell culture experiments with rat AR (Ikonen *et al*., 1994), we report here that the modulators of PKA affects transactivational activity of the yeast expressed receptor in ligand dependent manner.

Results

Activators of protein kinase-A signalling pathway influence ligand induced AR mediated gene expression

Rat AR upon transformation in *S. cerevisiae*, strain YCR1 showed DHT inducible β-galactosidase activity (Rana *et al*., 1998). Utilizing this system, we have examined the effect of the modulators of PKA pathway in influencing AR-mediated reporter gene transactivation. PKA activators (20 μM forskolin or 1 mM 8-Br-cAMP) or protein phosphatases inhibitor (100 nM okadaic acid) induced the reporter activity only in the presence of androgens (Figure 1). The extent of activation with forskolin in the presence of androgen was $\geqslant$1.5-fold compared to that seen with DHT alone (137.96(±39.02) Miller units, mean ± SD, $n = 22$, in response to 50 nM DHT). However, neither of them were able to activate the basal level of β-galactosidase activity in the absence of hormone. Furthermore, such effects of these modulators could be reversed by kinase inhibitor, 1-(5-isoquinolinsylsulphonyl)-2-methyl- piperazine (H7;

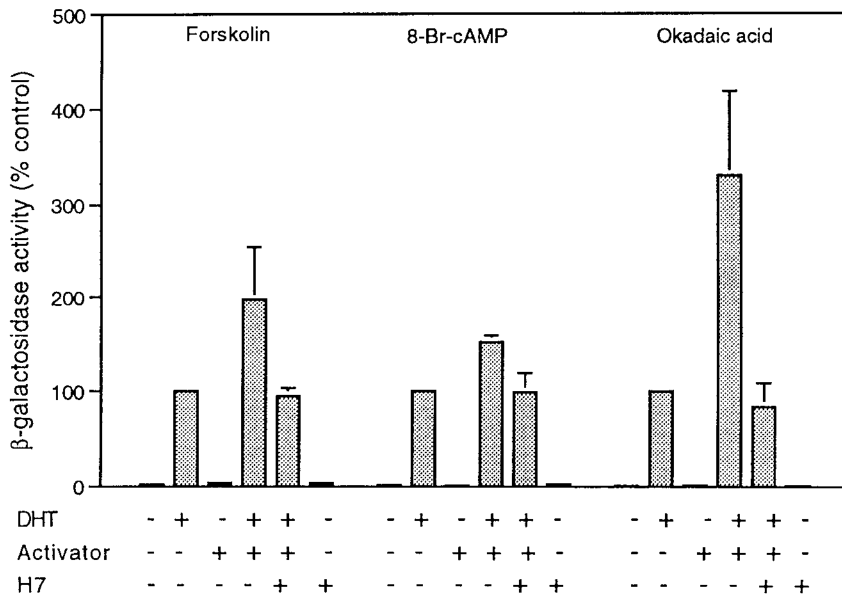


Figure 1. Stimulation of DHT-induced β-galactosidase activity by modulators of protein kinase-A. YCR1 cells containing pGAR were grown in SD/-Ura-Trp medium containing vehicle (1 % (v/v) ethanol) or 50 nM DHT in the presence (+) or absence (−) of activators (20 μM forskolin, 1 mM 8-Br-cAMP or 100 nM okadaic acid) and/or H7 (50 μM). β-Galactosidase activity is expressed relative to that achieved with pGAR in the presence of DHT only.

50 μM), suggesting the involvement of reversible phosphorylation events in the androgen-regulated transcriptional activation. These results therefore argue a synergistic influence of PKA pathway on ligand dependent AR-mediated gene expression.

To confirm that forskolin interaction with AR mediated signal transduction pathway is ligand dependent, we studied a receptor mutant (mHBD1-AR) which displays a high level of β-galactosidase activity in a ligand independent manner. Unlike wild-type AR, when YCR1 was transformed with this mutant receptor, reporter activity was unaffected, whether induced with or without DHT, both in the presence and absence of forskolin (Figure 2).

Forskolin does not affect receptor synthesis in YCR1 cells

The synergistic influence of forskolin on androgen-mediated transcriptional activation of the reporter gene could be an effect of increased receptor synthesis. To have an insight on this aspect, we monitored mRNA level synthesis of AR utilizing RT-PCR. The sensitivity of such a technique as opposed to a typical Northern analysis has been suggested to be a powerful alternative, particularly in situations where relatively low abundance of specific mRNA (Kahn & Chelly, 1994) are present. YCR1 cells transformed with AR were incubated with either DHT or forskolin, or with a combination of DHT and forskolin. Total RNA extracted from yeast transformants was reverse transcribed using random primers and AMV reverse transcriptase. PCR using reverse transcription products (different treatments or other controls) as templates with primers P1 and P2 indicated amplification of a ~500 bp fragment in all the samples transformed with AR, irrespective of the treatments. Southern analysis of the RT-PCR products using the *StuI-NdeI* fragment of rat AR as a probe, hybridized only with this ~500 bp band and showed its expression in all the samples (ethanol/DHT/forskolin/forskolin + DHT), except in control YCR1 cells not transformed with AR (Figure 3(a)). However, among different treatments, there was no significant difference in the expression at the mRNA level. To rule out the possibility that differential expression of ~500 bp fragment in AR-expressed cells was not an experimental artifact, PCR has also been carried out with mouse β-actin primers (P3 and P4) using the same reverse transcription products as templates. Southern hybridization of the ~543 bp PCR amplified products using yeast *act1* as a probe showed the expression of β-actin in

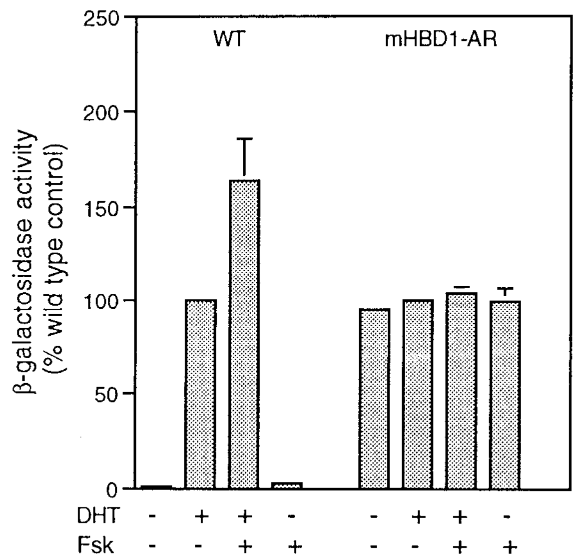


Figure 2. Forskolin does not affect reporter activity in an AR mutant exhibiting DHT independent transactivational activity. YCR1 cells were transformed with wild-type rat AR (pGAR) or mutant rat AR (mHBD1-AR). Cultures were grown with or without 50 nM DHT supplemented with 20 μM forskolin (Fsk). β-Galactosidase activity is expressed as a percentage of wild-type.

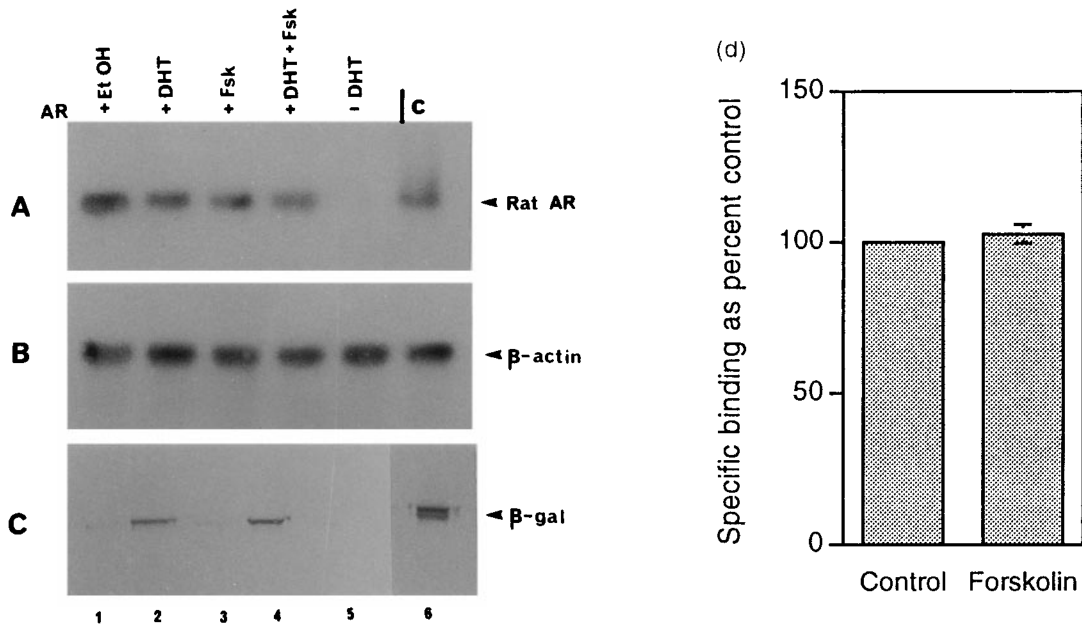


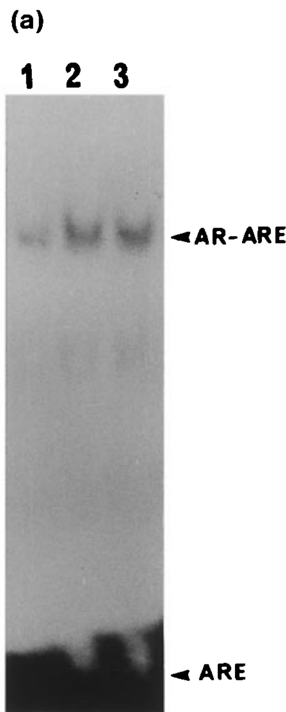
Figure 3. Forskolin does not affect rat AR mRNA synthesis or specific binding of androgen to yeast-expressed rat AR. YCR1 cells transformed with AR (+) were cultured in the presence of ethanol (lane 1), 50 nM DHT (lane 2), 20 μM forskolin (lane 3), 50 nM DHT and 20 μM forskolin (lane 4). Mock transformed YCR1 cells (-) were grown in the presence of 50 nM DHT (lane 5). Total RNA was extracted to carry out RT-PCR and cell extracts were prepared for Western blotting. Positive controls are indicated by C (lane 6). (a) Southern hybridization of RT-PCR products with primers P1 and P2 showing expression of a ~500 bp band in samples containing rat AR only. The blot was probed with the *Stu*I-*Nde*I fragment of rat AR. Using same primers, the PCR-amplified band from AR cDNA served as a positive control. (b) Southern hybridization of RT-PCR products with primers P3 and P4 showing expression of a ~543 bp band of β-actin in all samples. *Hin*dIII-*Hin*dIII fragment of yeast *act1* was used as probe. PCR amplified product using the same primers from *act1* cDNA served as a positive control. (c) Western blot analysis with yeast extracts using anti-β-galactosidase antibody. *E. coli* β-galactosidase enzyme was used as a positive control. (d) Whole cell binding assay to determine specific binding of [^{3}H]R1881 to rat AR-transformed YCR1 cells in response to forskolin treatment.

all samples including the control. However, there was no significant difference in the level of β-actin among the samples (Figure 3(b)). The results obtained, therefore, implicated that the mRNA level expression of AR was specific for YCR1 strain transformed with AR, and that forskolin alone or in combination with DHT had no effect on the amount of receptor synthesis. The corresponding β-galactosidase expression in response to different treatments was monitored by immunoblotting using a monoclonal antibody against the enzyme. As can be seen in Figure 3(c), forskolin alone was unable to induce β-galactosidase expression. Furthermore, forskolin treatment did not result in any significant alteration in [^{3}H]R1881-specific binding to the yeast expressed receptor as determined in whole cell binding assay (Figure 3(d)). This observation thus implicated that forskolin treatment did not alter the cellular content of AR protein capable of steroid binding.

Forskolin promotes AR binding to ARE, but subsequent interaction with the transcription machinery in the signal transduction process is androgen dependent

To evaluate the possibility of the alteration in the level of AR-ARE interaction, pGAR-transformed YCR1 cells were grown in the presence of forskolin (20 μM) or DHT (50 nM) for ten hours at 30 °C. Yeast extracts were prepared and gel retardation assays were carried out with ^{32}P-labelled ARE. Forskolin alone was able to promote binding of AR to ARE and there was no significant difference in the relative band intensities of receptor-DNA complexes formed in response to DHT or forskolin (Figure 4(a)). Even *in vitro* incubation of yeast extract with forskolin alone showed binding of AR to ARE in gel retardation assays (Figure 4(b)). The AR-ARE interaction due to forskolin treatment was specific, since only the cold ARE, but not any non-specific DNA sequence, competed with the receptor-DNA binding to any significant extent (Figure 4(b)). However, despite AR-ARE binding, forskolin alone was unable to induce any β-galactosidase expression (see Figure 3(c)) and the reporter activity was noticed only in the presence of DHT. Thus, our results indicate that although forskolin can initiate receptor-DNA binding, for the subsequent activation of the transcription machinery and induction of β-galactosidase activity, DHT is required in the process.

To determine whether the synergistic action of protein kinase activators has any relationship with the degree of ARE occupancy, we monitored DHT



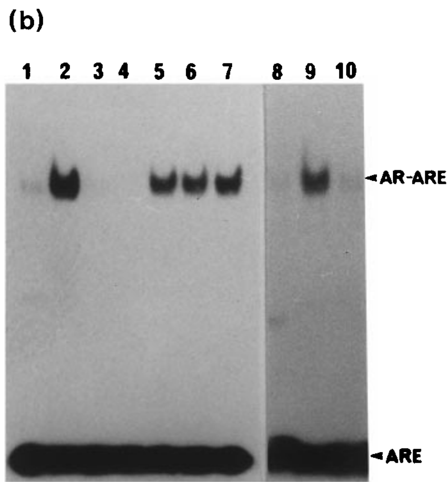


Figure 4. Forskolin alone initiates AR-ARE complex formation. (a) Yeast extracts were prepared from pGAR-transformed YCR1 cells grown in the presence of 1 % ethanol (lane 1), DHT (lane 2) or forskolin (lane 3) for ten hours. After harvesting, the cell extracts were prepared and incubated with ^{32}P-labelled ARE. Samples were then subjected to EMSA. (b) *In vitro* binding of pGAR-transformed rat AR to ^{32}P-labelled ARE following different treatments as described in Materials and Methods. EMSA was carried with these samples. YCR1 extracts incubated with 50 nM DHT (lanes 1 and 10); pGAR transformed YCR1 extracts incubated with 20 μM forskolin alone (lanes 2 and 9) or in combination of forskolin and 5 (lane 3) and 100-fold (lane 4) excess of non-radioactive ARE or in the presence of 100-fold excess of NFκB (lane 5) or in combination with 50 nM DHT in the presence of 100-fold excess of NFκB (lane 6) or DHT alone (lane 7) or 1 % ethanol alone (lane 8). Samples shown in lanes 8-10 are from a different experiment.

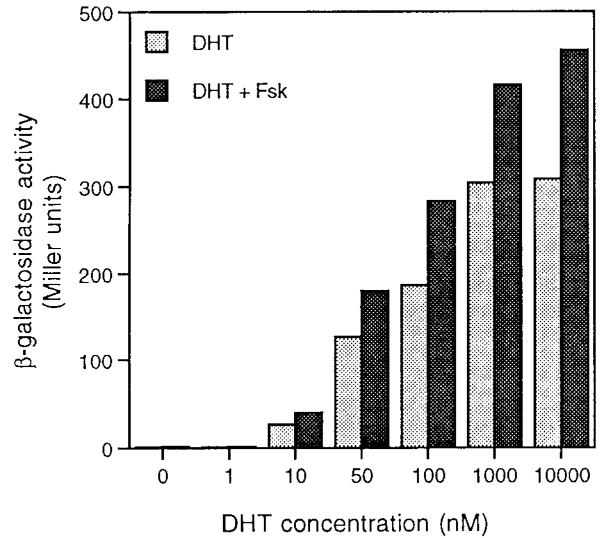


Figure 5. DHT-induced ARE occupancy of yeast expressed rat AR is unaffected by forskolin. YCR1 cells transformed with pGAR were treated to the indicated concentrations of DHT (1 nM to 10 μM) with or without 20 μM forskolin for ten hours, and β-galactosidase activity was monitored.

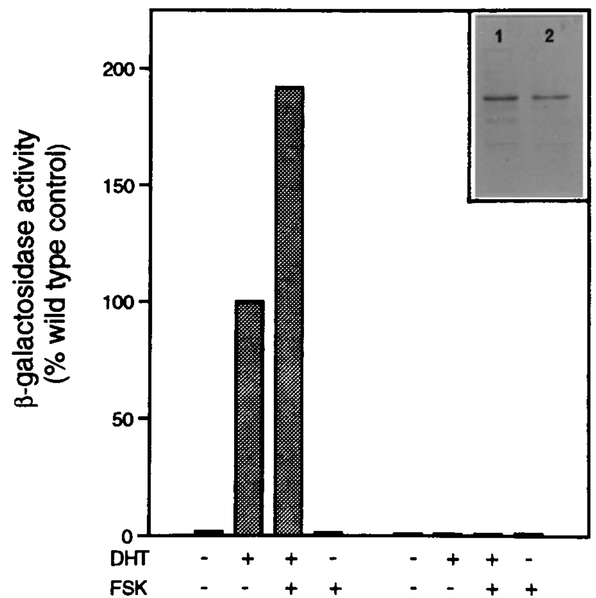


Figure 6. Intact DNA binding domain of the receptor is critical for mediating ligand-dependent forskolin action. YCR1 cells were transformed with wild-type AR (first group of columns) or C562G mutant rat AR (second group of columns). Cultures were treated with 1 % ethanol, 50 nM DHT or 20 μM forskolin alone or in combination as indicated. Data are expressed as the percentage of induction relative to β-galactosidase activity obtained with wild-type (pGAR) in the presence of 50 nM DHT. Inset, Western blot using anti-AR antibody showing the receptor expression in both wild-type (lane 1) and C562G (lane 2).

dose-dependent β-galactosidase activity in the presence of forskolin. A similar relative increase in reporter activity has been noticed in response to 20 μM forskolin (Figure 5) between the DHT concentrations of 1 nM to 10 μM and the EC_{50} value (concentration of the steroid at which 50 % of maximum transcriptional activation has been achieved) remained unaltered (45(±8) nM, $n = 5$). This result therefore indicates that the synergism is independent of the status of ARE occupancy.

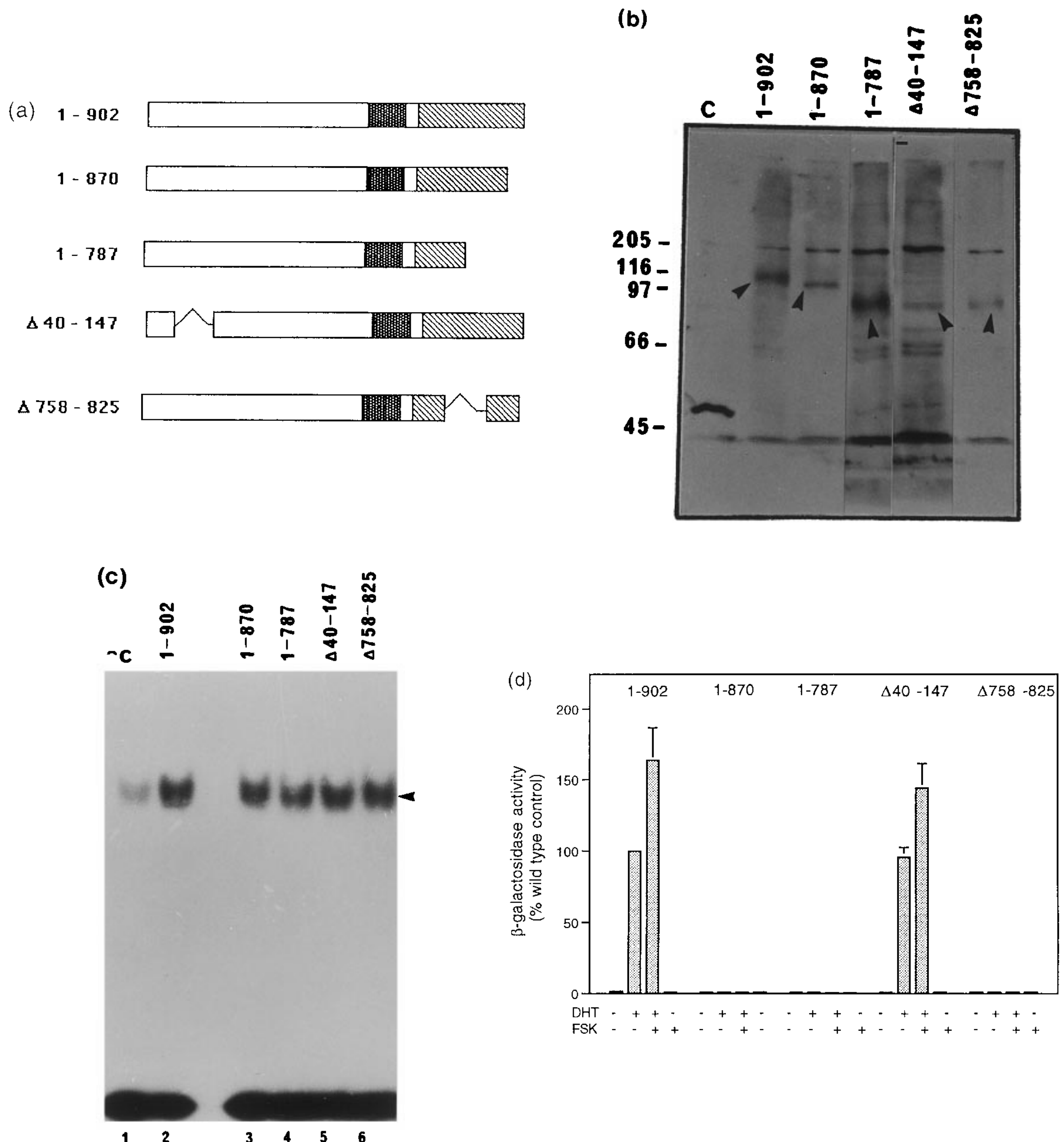


Figure 7. The entire steroid binding domain of the receptor is necessary for mediating the effect of forskolin. (a) Schematic representation of wild-type rat androgen receptor (1-902) and the receptor mutants (1-870, 1-787, Δ40-147 and Δ758-825). The numbers indicate the span of amino acid residues in the mutants; Δ followed by numbers indicate the deleted regions. (b) Immunoblot analysis of yeast-expressed rat AR deletion mutants with antibody 3777. Positions of the wild-type and mutants are indicated by arrowheads. C denotes the mock transformed control. (c) Electrophoretic mobility shift assay with AR deletion mutants following induction with 50 nM DHT. Mock transformed control treated with 50 nM DHT is indicated by C; the arrow denotes the AR-ARE complex. (d) Effect of forskolin on androgen-induced β-galactosidase activity with deletion mutants. The cells were treated with 1 % ethanol (−), 50 nM DHT or 20 μM forskolin (+). β-Galactosidase activity was expressed as percentage of wild-type induced with DHT.

DNA binding domain is not enough for mediating ligand dependent forskolin action on reporter gene transcriptional activation

Mutational studies revealed that alteration of amino acids in the first zinc finger region of the AR DNA binding domain led to transcriptional inactivation (Zoppi *et al.*, 1992). To verify this in our system, we created one such rat receptor point mutant, C562G, which failed to exhibit any reporter gene expression in response to DHT or forskolin alone or even in combination (Figure 6), although the receptor protein was expressed to the level of wild-type (Figure 6, inset). Since the synergistic activation of reporter activity by forskolin in our system was ligand dependent, it would be logical to presume that in addition to the DNA binding domain other receptor domain(s) might be involved in the process.

To identify such involvement of other receptor domain(s) in the synergism between DHT and forskolin in AR-dependent transactivation, we constructed several deletion mutants (Figure 7(a)) and expressed them in YCR1 utilizing the same 2 μ-based yeast expression vector pG1. Western blot using anti-receptor antibody 3777 showed expression of all mutant (1-870, 1-787, Δ40-147, and Δ 758-825) receptors (Figure 7(b)). Since the DNA binding domain of these mutants were unaffected due to deletion, as expected they exhibited binding to ARE when induced with DHT as observed in wild-type (Figure 7(c)). The binding of mutant receptors to DNA was specific, since these bands were abolished when competed with cold ARE but not with any non-specific DNA sequence (data not shown). As in the wild-type AR, synergistic activation of β-galactosidase activity by forskolin and DHT in combination was unaffected in the Δ40-147 N-terminal deletion mutant. However, all the ligand binding domain deletion mutants (1-870, 1-787, and Δ758-825), despite target DNA binding, did not show any DHT or forskolin plus DHT induced detectable reporter activity (Figure 7(d)). This was not unusual, since ligand-dependent TAF-2 function of the hormone binding domain of AR has already been shown to be affected by similar mutants (Moilanen *et al.*, 1997). Thus, our results indicate that in addition to the DNA binding domain, the entire hormone binding domain of the rat AR is required for exhibiting synergism between PKA activator and androgen.

Synergistic interaction of forskolin in AR-mediated signal transduction is inhibited by androgen antagonists

We have recently reported that anti-androgens are ineffective in exhibiting their antagonistic behaviour with yeast expressed rat AR (Rana *et al.*, 1998). To elucidate whether forskolin has any influence in the process, reporter activity has been monitored following induction of yeast expressed rat

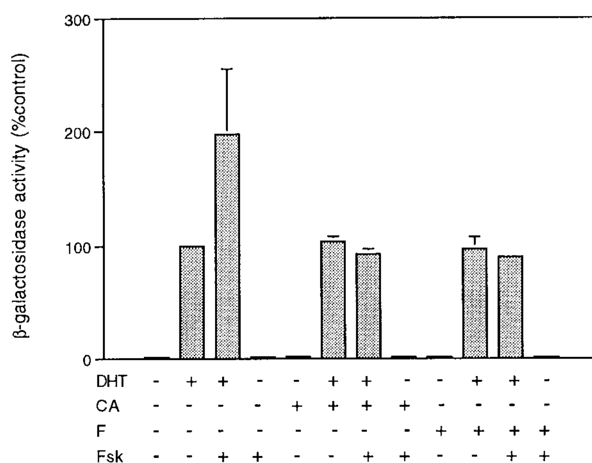


Figure 8. Antagonists inhibit forskolin dependent enhancement of the reporter activity. Following transformation with pGAR, YCR1 cells were treated with ethanol, 100 nM DHT or 20 μM forskolin either alone, or in combination with the two anti-androgens, CA (100 nM) or flutamide (1000 nM) as indicated by (+) or (−). β-Galactosidase activities were monitored and expressed as percentage of wild-type induced with DHT.

AR with DHT or steroidal antagonist, cyproterone acetate (CA) or non-steroidal antagonist, flutamide or in combination of hormone and anti-hormone. Both CA and flutamide were unable to induce any β-galactosidase activity even in the presence of forskolin. Furthermore, in conformity to our previous findings (Rana *et al.*, 1998), none of the anti-androgens at the tested concentrations were able to antagonize the DHT-dependent induction of reporter activity (Figure 8) and forskolin had no effect on influencing the anti-androgenic response of CA as well as flutamide. Surprisingly, enhancement of agonist-dependent reporter activity by forskolin has been abolished in the presence of CA or flutamide, and this is specific since no such effect has been observed with a non-specific steroid like progesterone (data not shown).

Discussion

Until recently, it was an established dogma that ligand binding is an absolute requirement for the activation of steroid receptors. It was thought that only a cognate ligand could bring about the necessary changes required for DNA binding followed by transcriptional activation. Therefore, it came as a big surprise when more and more evidence accumulated to suggest that steroid receptors could also mediate other extracellular signals, both in the presence and absence of cognate ligands (Power *et al.*, 1992; O'Malley *et al.*, 1995). Our results add to a growing body of evidence suggesting the existence of regulatory interplay between different cell signalling pathways. Steroid hormone receptors are phosphoproteins and hormone binding

has been reported to elicit a rapid increase in the extent of phosphorylation of many receptors (Moudgil, 1990; Orti *et al*., 1992). This suggests that the phosphorylation status and transactivation capability of the receptors are interlinked. Available reports addressing the influence of modulators of protein phosphorylation on androgen-AR-ARE mediated transcription, however, are rather incomplete and have yielded contradictory results (Ikonen *et al*., 1994; de Ruiter *et al*., 1995; Nazareth & Weigel, 1996). Such an aspect demands attention, especially in the clinical settings like androgen resistance and even in prostate cancer.

In order to analyse the complex mechanisms of this cross-talk, we focussed on establishing a heterologous expression system employing yeast as a host to capitalize on the power of yeast genetics. Such a yeast-based AR expression system has already been shown to recapitulate the regulatory machinery controlling androgen dependent transactivation (Purvis *et al*., 1991; Mak *et al*., 1994; Fang *et al*., 1996; Doesberg *et al*., 1997; Rana *et al*., 1998). Therefore, we wanted to know whether a similar system expressing rat AR (Rana *et al*., 1998) could be profitably utilized in unravelling the mechanisms of regulatory interplay between two signalling pathways. Three lines of evidence from our study argued that the synergistic interactions between PKA and rat AR-mediated signalling pathways are ligand dependent. First, exposure of YCR1 cells containing rat AR to activators of adenyl cyclase (forskolin and 8-Br-cAMP) in the presence of DHT, enhanced the reporter activity $\geqslant$1.5-fold compared to DHT alone (Figure 1). Second, okadaic acid, which is a potent inhibitor of protein phosphatases, also caused enhancement of androgen-dependent, AR-mediated transcriptional activation (Figure 1). Third, the cAMP-dependent protein kinase-A/C inhibitor, H7, completely reversed the effects of forskolin, 8-Br-cAMP or okadaic acid. All these modulators did not influence β-galactosidase basal activity. Furthermore, these modulators were without any effect when YCR1 was transformed with the expression vector, pG1, which indicates that the observed synergism is mediated by the rat AR. Thus, our results are in conformity with the observations in mammalian cell culture experiments with rat AR (Ikonen *et al*., 1994). Similar synergistic enhancement of ligand-dependent, receptor-mediated transactivation by PKA activators or protein phosphatase inhibitors (Figure 1) has also been observed in experiments with mammalian cells expressing human progesterone (Beck *et al*., 1992) and glucocorticoid (Moyer *et al*., 1993; Nordeen *et al*., 1994) receptors.

As far as ligand dependency is concerned, there exist some discrepancy in manifestation of such cross-talks between rat (Ikonen *et al*., 1994) and human AR (Nazareth & Weigel, 1996). However, it does not seem to be AR specific, since a similar difference has also been noticed with chicken (Denner *et al*., 1990; Power *et al*., 1992) and human (Beck *et al*., 1992) progesterone receptors. It seems possible that such dissimilar results could be due to factors like species specificity, cell lineage and promoter context of steroid induced reporter constructs (Nazareth & Weigel, 1996). Alternatively, it could be an intrinsic difference between rat and human androgen receptors. Such a possibility is not remote, since differences in the intrinsic properties between chicken and human progesterone receptors have also been noticed (Beck *et al*., 1992). However, it is apparent from our study that the yeast system faithfully recapitulates the interaction of PKA modulators and rat AR-mediated signalling pathways as seen in mammalian cells (Ikonen *et al*., 1994).

Mutational studies revealed that the intact DNA binding domain of the steroid receptor is necessary for transcriptional activation (Zoppi *et al*., 1992). This has further been supported by our results with the C562G mutant (Figure 6). Since the synergistic activation of reporter activity by forskolin in our system was ligand dependent, it would be logical to presume that in addition to the DNA binding domain, a hormone binding domain is also involved in the process. Moreover, TAF-2 in the hormone-binding domain of AR, which is essential for hormone dependent transactivation, functions quite actively in yeast compared to mammalian cells (Moilanen *et al*., 1997). None of the hormone binding domain deletion mutants (1-870, 1-787, and Δ758-825) show any detectable reporter activity when induced with DHT alone or in combination with forskolin and DHT (Figure 7(d)). Different levels of receptor expression (Figure 7(b)), on the other hand, were unable to account for the absence of such reporter activity, since all the mutant receptors bind to target DNA and intensity of the AR-ARE bands were indistinguishable from the wild-type (Figure 7(c)). Interestingly, ligand-dependent TAF-2 function of the hormone binding domain of AR has been reported to be abolished by similar mutants (Moilanen *et al*., 1997). In such a consequence, our results suggest that DNA and hormone binding domains of AR, which are mandatory for steroid-dependent transactivation, are also needed for the cross-talk between the two signalling pathways.

Forskolin alone or in combination with DHT had no effect on the amount of receptor mRNA synthesis (Figure 3(a)). Forskolin treatment also did not initiate changes in the cellular content of AR protein capable of [^{3}H]R1881 binding (Figure 3(d)), although it promoted specific binding of AR to ARE *in vivo* (Figure 4(a)). *In vitro* gel reterdation assays (Figure 4(b)) also showed such receptor-DNA binding in yeast extracts when incubated with forskolin. Considering other evidence we presented here and based on available reports (Beck *et al*., 1992; Rangarajan *et al*., 1992; Ikonen *et al*., 1994), forskolin *per se* imparting AR-ARE binding seems to be very unlikely. The only conceivable explanation of such an observation could be that the yeast extracts prepared by ammonium sulphate precipitation were not completely ATP depleted. In

fact, the precipitate was neither washed nor dialysed (see Materials and Methods). As a result, the presence of ATP in the extract at low concentration could not be ruled out. On the other hand, *in vivo* gel retardation assay did not show any apparent significant difference in the relative band intensities of receptor-DNA complexes formed in response to DHT or forskolin alone (Figure 4(a)) or DHT and forskolin in combination (data not shown). However, in the absence of androgen, forskolin was unable to interact with the transcriptional machinery to induce β-galactosidase activity (Figure 3(c)). In such a situation, this discrepancy subscribes to the fact that synergistic influence exerted by forskolin on the androgen-induced signalling pathway is mediated at a step after the receptor binds to DNA.

Phosphorylation of AR itself could lead to a change in either hormone binding or DNA binding, and forskolin may be promoting this event. Three phosphorylation sites have been identified in AR which are serine 81, 94 and 640 in the human receptor (Zhou *et al.*, 1995) corresponding with amino acid residues 65, 78, and 624 of the rat receptor. Since the synergistic activation of β-galactosidase activity by forskolin and DHT in combination was unaffected in the deletion mutant Δ40-147, it indicates that the two N-terminal domain phosphorylating residues (serine 65 and 78) are not involved in the process. In mutants like mHBD1-AR, where all three phosphorylation sites of the receptor are intact, reporter activity was unaffected upon forskolin treatment. Additionally, in immunoblots, no difference has been observed in the migration patterns between hyperphosphorylated and wild-type rat ARs (Ikonen *et al.*, 1994). It thus seems very unlikely that receptor phosphorylation *per se* would influence the event, rather a variety of receptor associated proteins of the hsp90-aporeceptor complex could be potential targets. Furthermore, it is well established that several transcriptional co-activators of steroid receptors like, ARA70 (Yeh & Chang, 1996), GRIP1 (Hong *et al.*, 1996), SNF2/SWI2 (Yoshinaga *et al.*, 1992) and SRC-1 (Onate *et al.*, 1995) are involved in the enhancement of the agonist dependent interaction of receptors with the basal transcriptional machinery (Manglesdorf & Evans, 1995) in mammalian cells as well as in yeast. The target for phosphorylation events may be one or more of these auxiliary proteins or co-activators which could result in a change in their activity. Available reports with human AR (Nazareth & Weigel, 1996) and other steroid receptors (Beck *et al.*, 1992; Moyer *et al.*, 1993; Nordeen *et al.*, 1994) also favour of such conclusions. At this juncture, it seems to be worth mentioning that anti-androgens, although unable to exert their antagonistic behaviour with yeast expressed rat AR, were able to attenuate the net-fold synergistic increase in DHT-mediated β-galactosidase activity by forskolin (Figure 8). It therefore is tempting to speculate that coupling of two different pathways could be mediated by such auxiliary proteins or co-activators, and anti-androgens, although unable to exert their antagonistic effect in yeast, are competent in blocking receptor activation by modulators of PKA.

Finally, our results clearly indicate that the steroid-dependent activation of rat AR by the modulators of kinase-phosphatase pathways could be recapitulated in yeast. In spite of several studies, the nature of interaction between these signalling pathways are essentially unknown. Therefore, recapitulating this phenomenon in yeast definitely provides us with an efficient means for genetic analysis of different aspects, towards a better understanding of the cross-talk between modulators of protein phosphorylation and steroid receptors.

Materials and Methods

Materials

Non-radioactive R1881(methyltrienolone) and [^{3}H]R1881 (85.5 Ci/mmol) were procured from New England Nuclear, USA. The [γ-^{32}P]ATP, [α-^{32}P]ATP and [α-^{32}P]dCTP were supplied by Bhaba Atomic Research Centre, India. Restriction/modifying enzymes and other molecular biological reagents were obtained either from New England Biolabs or Promega Corporation, USA. ECL Western blotting detection kit (Amersham, England), Geneclean kit (Bio 101, USA), poly(dA-dT) (Pharmacia, Sweden), protein molecular mass markers (Sigma Chemical Company, USA), Sequenase kit (version 2.0, Amersham, England), *Taq* DNA polymerase (Boehringer Mannheim, Germany), X-ray film (Eastman Kodak, USA) and yeast nitrogen base (Difco, USA) are commercially available. All other chemicals including steroids/antisteroids, forskolin, okadaic acid, etc. were procured from Sigma Chemical Company, USA. All oligonucleotides used in this study were custom synthesized from Ransom Hill, USA, except androgen/glucocorticoid response element (ARE/GRE, double-stranded oligonucleotide, referred as ARE) which was obtained from Promega, USA.

Antibodies

Anti-mouse AR polyclonal antibody 3777, which recognizes rat AR (Fang *et al.*, 1996), was a gift from Dr A. Caplan, Mount Sinai Medical School, New York, USA. Anti-β-galactosidase monoclonal and anti-mouse/anti-rabbit IgG-horseradish peroxidase conjugated antibodies were procured from Promega Corporation, USA.

Plasmid construction

Plasmid pGAR expressing the rat AR was constructed by subcloning the 2.8 kb *Eco*RI/*Pst*I fragment of rat AR cDNA (Chang *et al.*, 1988) into the *Bam*HI site of yeast expression vector pG1 (Schena *et al.*, 1991), following intermediate subcloning steps in pBluescript II SK(+)/KS(+), for incorporating *Bam*HI sites at both 5′ and 3′ ends of the insert as described (Rana *et al.*, 1998).

Different deletion mutants were generated following restriction digestions of rat AR with *Apa*I (Δ40-147), *Eco*RI/*Pst*I (1-787), *Sph*I (Δ758-825) and *Nde*I/*Pst*I (1-870). All these mutants were cloned in the *Bam*HI site of pG1 through intermediate subcloning steps in pBlue-

script II SK(+) or by *Bam*HI linker ligation. All deletions were confirmed by restriction digestion and Southern hybridization with suitable probes (data not shown). Yeast integrating vector pCR4 (~7 kb) containing GRE-*CYC1-lacZ* cassette was constructed by *Eco*RI digestion of plasmid pSX26.1 (Schena & Yamamoto, 1988), followed by self ligation after removing the 2 μ sequences.

Construction of yeast strain

Integrating vector pCR4 containing GRE-*CYC1-lacZ* cassette was linearized at the unique *Stu*I site present in its *URA3* coding sequence. Upon transformation into the protease-deficient yeast strain BJ 5460, it integrated at the defective *ura3-52* locus by homologous recombination. Transformants exhibiting uracil prototrophy were selected and integration was further confirmed by Southern hybridization using the β-galactosidase (832 bp *Bam*HI-*Cla*I fragment) and the *ura3* (456-bp *Pst*I-*Stu*I fragment) as probes (results not shown). The integrated strain designated as YCR1 was used in this study.

Generation of mutants

PCR was employed to generate C562G (cysteine is replaced by glycine at amino acid residue 562), a point mutant in the DNA binding domain of rat AR. Two forward primers, W (5′ GCAAGCCAGGAGGGTGACT 3′), X (5′ TCACTTGTGGCAGCGGCA 3′) and two reverse primers, Y (5′ AAGACCTTGCCGCTGCCACA 3′), Z (5′ GGG TCCTCAGTGGGGCTACC 3′), were synthesized. Base mismatch (underlined bases) for the desired mutations were incorporated in primers X and Y. To generate the mutant, two sets of primary and one set of secondary PCR reactions were carried out following a method described by Ho *et al*. (1989), using the gel purified rat AR (~2.8 kb) as template. Primary reactions were carried out with primers W/Y and X/Z, while for secondary PCR primers W and Z were used. Thus, the C562G mutation was contained within the amplified ~497 bp fragment of the rat AR which is having unique *Hin*dIII and *Sac*I sites. Secondary PCR product was Klenowed, kinased and concatamerized. The resulting multimers were digested with *Hin*dIII and *Sac*I and substituted for the corresponding wild-type fragment in the rat AR backbone. Mutations were confirmed by sequencing.

To generate a diverse library of variant sequences over the ~494 bp region primers P1 (5′ GTGGGCCA-AGGCCTT 3′) and P2 (5′ ACGCTCACCATATGGGACT 3′), corresponding to rat AR nucleotide sequences 2099-2113 and 2585-2610, respectively (Chang *et al*., 1988), were utilized and random PCR mutagenesis was carried out under the conditions specified by Cadwell & Joyce (1995). The mutagenized pool of amplified DNA was gel purified and a library of hormone binding domain mutants of rat AR cDNA was generated by substituting the mutagenized *Stu*I-*Nde*I fragment for the corresponding wild-type region following *in vivo* gap-repair in YCR1 (Rothstein, 1991). Transformants were selected on SD/-Ura-Trp plates (see below) and then replica plated onto the same medium containing 1 % ethanol/50 nM DHT/1 μM CA/100 nM flutamide/100 nM progesterone. The AR hormone binding domain mutants were identified by screening for an altered response to different ligands. One such mutant which displays ligand independent activation of the *lacZ* reporter gene has been designated as mHBD1-AR and used in this study. Restriction digestion following back extraction of mHBD1-AR DNA from YCR1 revealed loss of both *Stu*I and *Nde*I sites. Hence, it is a multiple mutant. Although we observed mRNA level expression of mHBD-1 and the construct size is at par with the wild-type receptor, we did not observe any band in Western blot utilizing anti AR antibody 3777.

Transformation, growth and culture of yeast cells

Basic methods for yeast manipulations were carried out as described by Rose *et al*. (1990). YCR1 was transformed with pGAR (1 μg DNA) and appropriate colonies were selected on plates. Liquid selection medium (SD medium) containing 0.67 % (w/v) yeast nitrogen base, 2 % (w/v) dextrose, and required supplements except for relevant marker (uracil and/or tryptophan) was used for culture (30 °C at 200 rpm).

Preparation of yeast extract

All operations were carried out at 4 °C. Yeast lysates were prepared by disrupting cells (A_{600} at harvesting = 1.0) with glass beads in a cell disrupter (B. Braun Instruments) under CO_2 in HEDG buffer (20 mM Hepes (pH 7.9), containing 0.5 mM dithiothreitol, 0.2 mM EDTA, 30 mM KCl, 20 % (v/v) glycerol) and protease inhibitor cocktail (cell wet mass/volume = ~1 gm/5 ml). This was followed by low speed centrifugation (~16,000 *g* for 30 minutes). The supernatants, when necessary, were subjected to 50 % ammonium sulphate precipitation. Following centrifugation, the supernatant was aspirated and the precipitate was dissolved in HEDG buffer. Protein content of concentrated yeast extracts were determined (Bradford, 1976) and were adjusted to ~1 mg/ml before use in gel retardation assays or in Western blots.

Hormone induction and β-galactosidase assay

For hormone induction, saturated overnight cultures were diluted to 1:10 (A_{600} = ~0.2, ~5 × 10^6 cells/ml) in fresh selection medium, treated with DHT or vehicle (1 % (v/v) ethanol) or androgen antagonists (cyproterone acetate/flutamide) or other modulators (forskolin/8-Br-cAMP/okadaic acid/H7) alone or in combination with DHT and grown further for ten hours (A_{600} = ~1.8 to 2.0). In our experimental conditions, none of the treatments affected the growth rate of yeast cells. Quantitative β-galactosidase measurements in response to different treatments were carried out using a slight modification of the procedure by Schena *et al*. (1989). Briefly, 1.0 ml of culture was transferred to a microfuge tube, washed, pelleted and resuspended in 200 μl of Z buffer (60 mM Na_2HPO_4 $7H_2O$, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ $7H_2O$, 50 mM β-mercaptoethanol, pH 7.0). Cells were permeabilized by adding 50 μl chloroform and 20 μl of 0.1 % (w/v) SDS followed by vortexing for 30 seconds. Microfuge tubes containing permeabilized cells were then placed at 30 °C for five minutes for temperature equilibration. Following addition of *O*-nitrophenyl β-D-galactopyranoside (ONPG) solution (700 μl of 2 mg/ml stock in Z buffer), tubes were incubated for five to ten minutes. The reaction was stopped by the addition of 1 M Na_2CO_3 (500 μl) and a A_{420} reading of the supernatant was taken. The β-galactosidase activity was expressed in Miller units (Miller, 1992) and was calculated as 10^3 times the change in A_{420} (hydolysis of ONPG) divided by the pro-

ducts of duration of the assay (minutes), culture volume (ml) and cell density (A_{600}/ml).

Whole cell hormone binding assay

The whole cell binding assay was essentially as described by Fang *et al*. (1996). Briefly, yeast cells were grown to early log phase ($A_{600} = \sim 0.2$) and preincubated at 30 °C in 1 ml aliquots for 30 minutes; 100 nM [^{3}H]R1881 was added to the cultures in the presence or absence of a 500-fold excess of DHT. The cells were further incubated for three hours and collected on 25 mm GF/C filters using a 12-well cell harvester (Millipore). Cells were washed three times with 1 ml of sterile water, and [^{3}H]R1881 binding to yeast expressed rat AR was determined by counting in a scintillation counter with 5 ml scintillation fluid. Non-specific counts were subtracted from total counts in calculating specific binding (in dpm).

Electrophoretic gel mobility shift assay (EMSA)

Concentrated yeast extracts (following precipitation in 50 % ammonium sulphate, pellets obtained were dissolved in the HEDG buffer, protein concentration = ~1 μg/μl) were used for gel retardation assays for *in vitro* studies (Rana *et al*., 1998). The lysate was incubated with DHT and/or forskolin or vehicle for two hours at 0 °C. For *in vivo* studies, saturated overnight cultures were diluted ($A_{600} = \sim 0.2$), treated with DHT/forskolin/ethanol, grown further for ten hours ($A_{600} = \sim 1.8$ to 2.0) and then harvested for the preparation of concentrated yeast extracts as mentioned above. The DNA binding reaction was performed at 23 °C by incubating steroid/forskolin bound lysates (15 μl) with [γ-^{32}P]ATP-labelled ARE (5′ TCGACTGTACAGGATGTTCTAGCTA CT 3′), poly(dA-dT) (2 μg) and bovine serum albumin (80 μg) for 15 minutes. The samples were then resolved through native 5 % polyacrylamide gel electrophoresis (80 volts) using 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 0.1 mM EDTA, pH 8.4) followed by gel drying and autoradiography.

SDS/polyacrylamide gel electrophoresis and Western blotting

Yeast extracts (for β-galactosidase 80 μg protein/slot and for AR 25 μg protein/slot from the ammonium sulphate-precipitated fraction) were resolved in SDS-8 % PAGE and transferred to nitrocellulose membrane (0.45 μm) in a transblot apparatus (Bio-Rad) using Tris-glycine buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol, pH ~8.0). The transfer was carried out at 100 V for four hours at 4 °C and processed as described (Chakraborti *et al*., 1991). The β-galactosidase expression was monitored using anti-β-galactosidase monoclonal and anti-mouse IgG-horseradish peroxidase conjugated antibodies, and blots were developed with diaminobenzidine substrate solution (30 mg diaminobenzidine, 600 μl methanol, 30 mg imidazole in 30 ml of TBS containing 10 μl H_2O_2). Receptor expression was monitored using anti-AR 3777 antibody and the blots were processed with ECL detection system following manufacturer's recommended protocol.

RT-PCR

Cultures incubated with forskolin or DHT or in combination were treated with cyclohexamide (50 μg/ml of culture) for 30 minutes before harvesting. RNA from these cultures were extracted following the hot phenol method (Schmitt *et al*., 1990), and then treated with RNase free RQ1 DNase (0.1 unit/μl for one hour at 37 °C). The quality of the RNA preparation was checked by running an aliquot of the preparations in formamide-agarose gels. Total RNA (2 μg heated 95 °C for five minutes immediately before use) was used to set up each RT reaction (total volume of 50 μl) following standard protocol using AMV reverse transcriptase (25 units/reaction) and random hexamers (0.2 μg/reaction). The reaction was carried out at 42 °C for one hour. The RT products were precipitated in ethanol and dissolved in autoclaved distilled water (50 μl). To carry out PCR with RT products, two sets of primer (set 1, P1 and P2; set 2, control mouse β-actin primers: forward P3: 5′ GTGGGCCGCTCTAGGCACCA 3′ and reverse P4: 5′ CTC TTTGATGTCACGCACGATTTC 3′) were used. Aliquots (10 μl/25 μl reaction volume) of RT products along with necessary ingredients (primers, dNTPs, 1× buffer and *Taq* DNA polymerase) were mixed. PCR was initiated in a MJ Research Minicycler with a denaturation step (94 °C for three minutes) and carried out for 30 amplification cycles (45 seconds denaturation at 94 °C, 45 seconds annealing at 55 °C and 45 seconds of extension at 72 °C), followed by the final extension step for five minutes at 72 °C. PCR and RT-PCR products were separated on 1 % (w/v) agarose gels and were transferred to nylon membranes for Southern hybridization.

Southern hybridization

Southern/colony hybridization was carried out following standard protocols (Sambrook *et al*., 1989) using [α-^{32}P]dCTP/[γ-^{32}P]ATP-labelled probes.

Reproducibility of experiments

All experiments have been carried out with colonies obtained in two independent transformations. RT-PCR, Southern and Western blots and EMSA experiments were repeated at least twice with one colony from independent transformations. The β-galactosidase activity was monitored at least three times with two randomly selected colonies obtained in different transformations. Data are expressed as mean ± SD, except in Figure 5 which is a representative experiment. Statistical analyses have been carried out by Students' *t*-test and $p < 0.05$ has been considered as minimum level of significance.

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