

Inappropriate Activation of the Androgen Receptor by Nonsteroids: Involvement of the Src Kinase Pathway and Its Therapeutic Implications

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Abstract

The inappropriate activation of androgen receptor (AR) by nonsteroids is considered a potential mechanism in the emergence of hormone-refractory prostate tumors, but little is known about the properties of these “pseudoactivated” AR. Here, we present the first comprehensive analysis closely examining the properties of AR activated by the neuropeptide bombesin that distinguish it from androgen-activated AR. We show that bombesin-activated AR (a) is required for bombesin-induced growth of LNCaP cells, (b) has a transcriptional profile overlapping with, but not identical to, androgen-activated AR, (c) activates prostate-specific antigen by preferentially binding to its proximal promoter, and (d) assembles a distinct coactivator complex. Significantly, we found that Src kinase is critical for bombesin-induced AR-mediated activity and is required for translocation and transactivation of AR. Additionally, we identify *c-Myc*, a Src target gene, to be activated by bombesin and a potential coactivator of AR-mediated activity specific to bombesin-induced signaling. Because Src kinase is often activated by other nonsteroids, such as other neuropeptides, growth factors, chemokines, and cytokines, our findings have general applicability and provide rationale for investigating the efficacy of the Src kinase pathway as a target for the prevention of relapsed prostate cancers. (Cancer Res 2006; 66(21): 10449-59)

Introduction

Prostate cancer is the most common noncutaneous malignant transformation in American men. The initial emergence of prostate cancer is typically androgen dependent, treatable with androgen ablation therapy. However, in the majority of cases, tumors reemerge as androgen independent and highly aggressive and are associated with most prostate cancer-related deaths. Although androgen is suppressed under androgen ablation therapy, hormone-refractory tumors continue to express the androgen receptor (AR) and androgen-regulated genes (ARG; refs. 1, 2), and it has been suggested that relapsed tumors exhibit a “partial” or “inappropriate” reactivation of AR. Several nonmutually exclusive mechanisms have been proposed to account for inappropriate AR activation. Some androgen-independent tumors contain AR mutations that

can lead to the activation of AR by other steroids; however, these comprise a small percentage of relapsed tumors (3). Others carry overexpressed AR or cofactors (1, 2) as well as genes involved in androgen metabolism (4), which presumably sensitizes the cells toward low levels of androgen. In this report, we focus on another major mechanism under active investigation—the inappropriate activation of AR by nonsteroids through intracellular signaling pathways.

Various factors have been identified as ligand-independent activators of AR. These include growth factors [epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I; ref. 1)], interleukins [IL-6 (5)], neuropeptides [gastrin-releasing peptide (GRP) and neurotensin (6)], and chemokines [IL-8 (7)]. Although these factors differ in the initiation of the signaling pathways, they share the common trait of inducing phosphorylation cascades and activating tyrosine kinases, such as Src kinase, and other serine/threonine kinases. Phosphorylation of AR *in vivo* is required for its activation (1), and it has been suggested that further phosphorylation of AR by kinases can lead to its activation in the absence of ligand similar to mitogen-activated protein kinase (MAPK) modification of the estrogen receptor (ER; ref. 8).

Of growing interest is G protein-coupled receptor (GPCR) signaling by factors secreted by neuroendocrine cells. An increase in neuroendocrine cells present in the prostate has been associated with androgen ablation therapy (9), concomitant with an increase in soluble factors, such as GRP, neurotensin, serotonin, IL-8, and IL-6, which are secreted by neuroendocrine cells (10). Elevated expression of GPCRs for bombesin (homologous to GRP) and neurotensin has been found in prostate cancer biopsies (11). Receptors for GRP/bombesin, neurotensin, and IL-8 are found in both androgen-dependent (LNCaP) and androgen-independent (PC3 and DU145) prostate cancer cells (10) and are involved in an autocrine loop that stimulates growth in androgen-independent cells (12–14). We previously showed that bombesin, neurotensin, and IL-8 could activate AR and induce androgen-independent growth of LNCaP cells (7), implicating them as conspirators in the transition from an androgen-dependent to androgen-independent state.

Activation of AR requires the assembly of a transcriptional complex at the promoter, which includes AR, coactivators, and RNA polymerase II (Pol II; ref. 15). Among the various classes of coactivators that have been identified, the p160/SRC family of coactivators has been of significant interest in prostate cancer. These coactivators, which include SRC-1, SRC-2/GRIP-1/TIF-2, and SRC-3/ACTR/AIB1/RAC3/TRAM1, interact with AR in both a ligand-dependent and ligand-independent manner (1, 2). Their overexpression has been implicated in ligand-independent activation of AR and in the progression of prostate cancer.

In the present report, we describe the comprehensive analysis of androgen-independent activation of AR by the neuropeptide

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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bombesin. Our studies reveal for the first time that bombesin-activated AR assembles a set of cofactors and targets chromosomal sites distinct from androgen-bound AR and that this process involves oncoproteins c-Src and c-Myc in the signaling pathway.

Materials and Methods

Cell lines and reagents. LNCaP and PC3 cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Omega Scientific, Inc.). 5 α -Dihydrotestosterone (DHT), bombesin, GRP, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. Casodex was a gift from Dr. Xu-Bao Shi (UC Davis Cancer Center, University of California-Davis Medical Center, Sacramento, CA). Pyrazolopyrimidine (PP2) and SU6656 were obtained from Calbiochem.

Cell proliferation assay. LNCaP cells were hormone deprived in phenol red-free RPMI 1640 supplemented with 5% charcoal-dextran stripped (CDS) serum for 3 days, and then 5 \times 10³ cells per well were plated in 96-well plates in RPMI 1640 plus 0.5% CDS overnight. Cells were treated and proliferation was monitored by adding filtered MTT to cells at a final concentration of 500 μ g/mL and incubated at 37°C for 2 hours and crystals were dissolved with 10% SDS. Absorbance was read at 570 nm on an EMAX precision microplate reader (Molecular Devices).

For the RNA interference (RNAi) experiment, cells were infected with adenovirus after being hormone deprived for 3 days and then treated with agonist 48 hours after infection. The construction and purification of adenovirus carrying short hairpin RNA (shRNA) against AR (AR#2) was described previously (16). Viral titers were determined using 293 cells by end point cytopathic effect assay and/or Adeno-X Rapid Titer kit (Clontech). Western blot analysis of AR protein (AR#2) knockdown was published previously (16).

Immunofluorescent staining of AR localization. Immunofluorescent staining was done as described previously (17). Primary antibodies used for staining were rabbit polyclonal α -AR (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for endogenous AR or mouse monoclonal α -HA (HA.11) antibody (Covance) for AR-transfected cells. Secondary antibodies used were either α -rabbit or α -mouse Alexa Fluor 647 F(ab)₂ fragment of goat IgG (H+L; Invitrogen). Immunofluorescent cells were visualized using an Olympus BX61 motorized reflected fluorescence microscope system with an AMCA filter (excitation, 345 nm/emission, 445 nm) for 4',6-diamidino-2-phenylindole (DAPI) staining and Cy5 filter (excitation, 649 nm/emission, 668 nm) for AR localization using the SlideBook4.1 software (Intelligent Imaging Innovations).

Transactivation and luciferase reporter assays. ARE-Luc (6), ARE-I/II-Luc and ARE-III-Luc (18), and PSA6.0-Luc (19) reporter plasmids and transactivation assays (6) were described previously, except that cells were transfected with Effectene (Qiagen) according to the manufacturer's protocol using pRL-SV40 as an internal transfection control.

Microarray analysis. LNCaP cells used for DHT or bombesin treatment were grown to 70% to 75% confluency and then androgen deprived for 3 days in phenol red-free RPMI 1640 plus 5% CDS, serum starved overnight, and then treated for 6 hours. Total RNA was extracted twice using Trizol reagent and protocol (Invitrogen). RNA was submitted to the UC Davis Cancer Center Gene Expression Resource facility. Microarray analysis was done using Affymetrix Human Genome U133A (HG-U133A) GeneChip arrays (Affymetrix, South San Francisco, CA) as described previously (20). Analysis of the expression data from the bombesin-treated cells versus the vehicle-treated cells and from the DHT-treated cells versus the vehicle-treated cells was conducted using DNA-Chip Analyzer software (21). Data were filtered to select for transcripts having a fold change in expression of \geq 1.5-fold and a difference in expression between experimental and baseline groups of \geq 75. Statistically significant differential expression changes were selected based on attaining *P*s of \leq 0.05.

Western blot analysis. LNCaP cells were grown to confluency, serum starved overnight, and treated. Immunoblotting was done as described previously (22). Membranes were incubated in primary antibody [rabbit polyclonal α -phosphorylated Src Tyr⁴¹⁶ or rabbit polyclonal α -Src (36D10);

Cell Signaling Technology] overnight followed by incubation with a 1:10,000 dilution of horseradish peroxidase-goat α -rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) secondary antibody.

Reverse transcription-PCR analysis. RNA was isolated as described in Microarray section. Complimentary DNA synthesis and subsequent PCR were described previously (17). Sequences for each primer set are as follows: KLK2, 5'-CATCGAACCAGAGGAGTCTTGC-3' (forward) and 5'-GAAGCA-CACCATTACAGACAAGTGG-3' (reverse); KLK3, 5'-AACCAGAGGAGT-TCTTGACCCC-3' (forward) and 5'-GACGTGATACCTGAAGCACACC-3' (reverse); KLK4, 5'-GTTTCCAGAACTCCTACCCATCG-3' (forward) and 5'-AGGGCACTGCGAAGCAATG-3' (reverse); NKX3.1, 5'-CCAGCAGAAAAGG-CAGGGTAG-3' (forward) and 5'-TGGGAAAGCAAGATGGATTG-3' (reverse); TMEM16, 5'-TCCCTGTCTCATTGGTCGTC-3' (forward) and 5'-CGTGAAAG-CATTGGAATCAGCAC-3' (reverse); TMPRSS2, 5'-GAAAGGGAAGACCTCA-GAAGTGC-3' (forward) and 5'-CGATAAATCCAGTCCGTGAATACC-3' (reverse); SARG, 5'-CCAATGAACACAGAAGGGAGGC-3' (forward) and 5'-GG-GAACTCAATGGACAACACTG-3' (reverse); GREB1, 5'-GCGACCATCGGC-TTTAGGTATC-3' (forward) and 5'-TTCAGTTCCACAGAGGTAAGG-3' (reverse); IGFBP3, 5'-AACAGGAAGGAGGAATGGCTG-3' (forward) and 5'-AGGCAGAAATGTCTTGAGACCCC-3' (reverse); ZBTB16, 5'-CACACAGGT-GAGAAGCCCTTTG-3' (forward) and 5'-TTCTGCATGGAGGAGGCTG-3' (reverse); CAMKK2, 5'-GAGGCTTTGTGCTTATGAGAGTG-3' (forward) and 5'-AAGTTGACGGTGGTAATGGCTG-3' (reverse); MYC, 5'-ACCGAGGA-GAATGTCAAGAGGC-3' (forward) and 5'-CGTCGTTCCGCAACAAGTC-3' (reverse); GRB10, 5'-GGACGAAGTTGAGAAGGAAAACG-3' (forward) and 5'-TGACATCACAATCTACCGCCAG-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCTGTGCTGTA-3' (reverse).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were described previously (7), except that complexes were immunoprecipitated with 5 μ g of rabbit polyclonal α -AR (PG-21; Upstate), 10 μ g of rabbit polyclonal α -ACTR (H-270), SRC-1 (M-341), TIF-2 (GRIP-1 M-343), DAXX (M-112), CBP (A-22), MYC (N-262), or Pol II antibody (Santa Cruz Biotechnology). Androgen-responsive element (ARE)-I (proximal) primer sequences are 5'-TCTGCCTTTGTCCCTAGAT-3' (forward) and 5'-AACCTTCATTCGCCAGGACT-3' (reverse), and ARE-III (enhancer) primer sequences are 5'-CATGTTACATTAGTACACCTTGCC-3' (forward) and 5'-TCTCAGATCCAGGCTTGCTTACTGTC-3' (reverse).

Results

AR involvement in bombesin-induced growth of prostate cancer cells. Previously, we reported that bombesin was able to promote the growth of LNCaP cells in the absence of androgen (Fig. 1A; ref. 6). Although we can show that LNCaP cells respond to bombesin at a wide range of concentrations, we have used the upper range in our signaling studies, as others have done (23, 24), to account for the effect of neutral endopeptidase expressed at the surface of LNCaP cells, which cleaves and inactivates neuro-peptides (25). We were interested in determining if AR played a role in bombesin-induced growth of LNCaP cells or whether growth occurred by an AR-independent process. To test this, we used a RNAi-based approach to suppress AR expression. LNCaP cells were infected with an adenoviral vector expressing shRNA targeted against AR and then treated with bombesin and assayed for growth. As shown in Fig. 1B, cells infected with RNAi against AR had significantly reduced growth in response to bombesin compared with cells infected with negative control shRNA against green fluorescent protein (GFP). We further confirmed these findings using the nonsteroidal AR antagonist Casodex and found that LNCaP cells that were pretreated with Casodex did not respond to bombesin stimulation (Fig. 1C). These data suggest that AR is involved in bombesin-induced growth of LNCaP cells.

Bombesin-induced translocation of AR. Typically, binding of its ligand triggers translocation of AR from the cytoplasm to the

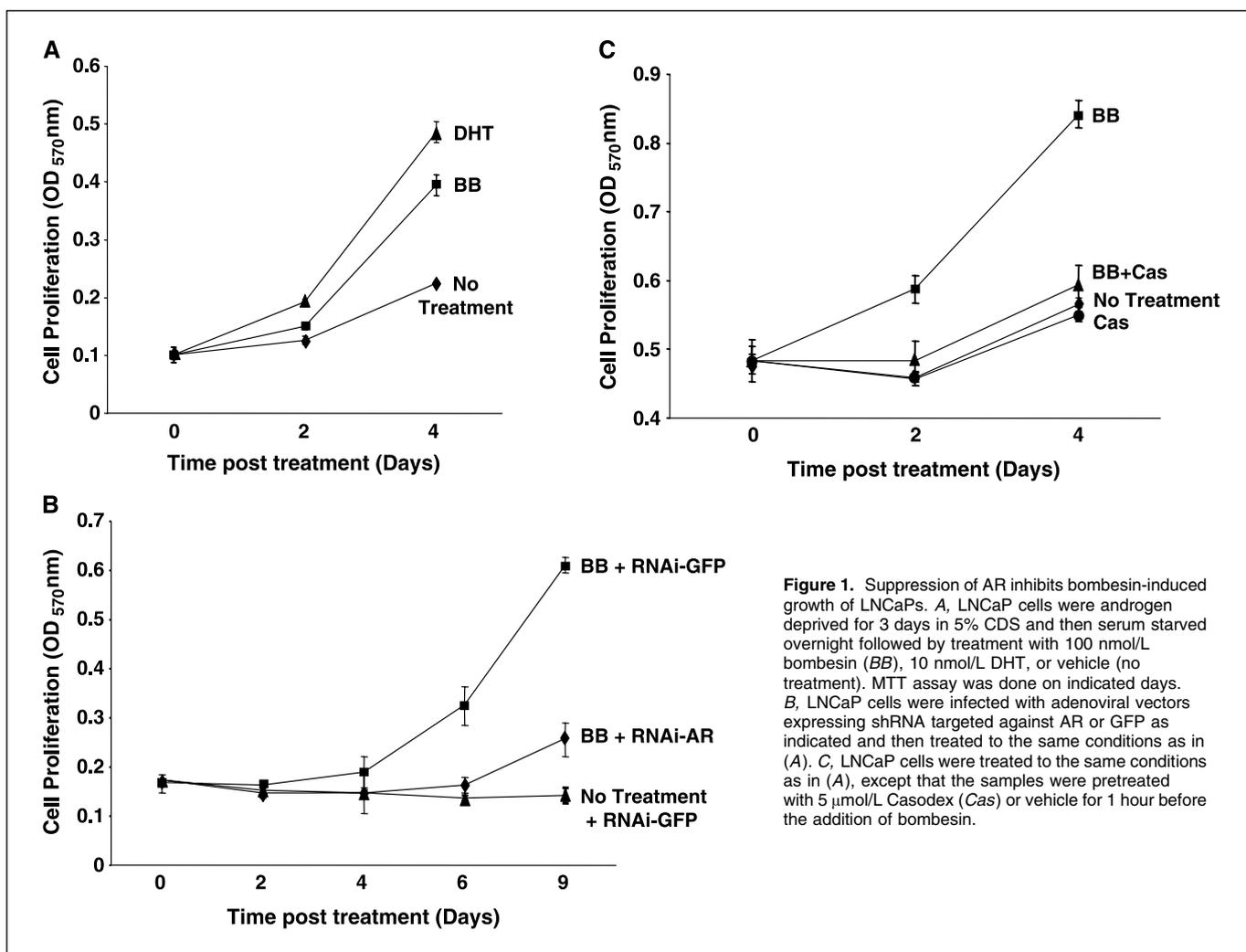


Figure 1. Suppression of AR inhibits bombesin-induced growth of LNCaPs. **A**, LNCaP cells were androgen deprived for 3 days in 5% CDS and then serum starved overnight followed by treatment with 100 nmol/L bombesin (BB), 10 nmol/L DHT, or vehicle (no treatment). MTT assay was done on indicated days. **B**, LNCaP cells were infected with adenoviral vectors expressing shRNA targeted against AR or GFP as indicated and then treated to the same conditions as in (A). **C**, LNCaP cells were treated to the same conditions as in (A), except that the samples were pretreated with 5 μ mol/L Casodex (Cas) or vehicle for 1 hour before the addition of bombesin.

nucleus, where it subsequently does its role as transcription factor. Bombesin-mediated AR signaling is not as obvious because bombesin interacts with cognate extracellular receptors and neither enters the cell nor binds to AR. We therefore wanted to verify AR translocation into the nucleus in response to bombesin treatment. LNCaP cells treated with either bombesin or DHT were processed for immunofluorescent staining of AR. As expected, AR was confined mainly to the cytoplasmic compartment in untreated cells, whereas AR was exclusively nuclear localized in the DHT-treated cells (Fig. 2A). Bombesin-treated cells also showed mainly nuclear staining, although some AR was still seen in the cytoplasm. We did observe that the overall intensity of AR staining was higher in the DHT-treated cells compared with the bombesin-treated cells presumably due to the stabilization of AR on ligand binding (26).

To verify that these results were not due to the T877A mutation present in AR found in LNCaP cells, we transfected LNCaP or PC3 cells with hyaluronic acid (HA) epitope-tagged wild-type AR and then treated with either bombesin or DHT followed by immunofluorescent staining for HA. In both cell lines, the untreated sample showed AR localized throughout the cell with the most intense staining seen in the cytoplasm (Fig. 2B and C, 1-3). We did not see exclusivity of AR in the cytoplasm most likely due to the overexpression of AR. On treatment with either bombesin or DHT, AR

was relocated entirely to the nucleus, confirming that bombesin induces translocation of AR.

Bombesin-induced ARGs. We previously reported that bombesin induced AR transactivation of ARE-driven promoters (Fig. 3A; ref. 6). Having shown that bombesin has the capacity to mediate AR nuclear translocation and transactivation, we next wanted to extend this by defining the bombesin-induced AR transcriptional program. To this end, we did genome-wide microarray expression profiling with Affymetrix HG-U133A GeneChip oligonucleotide arrays to determine differentially expressed genes in LNCaP cells treated with either bombesin or DHT. Analysis revealed that 72 genes were similarly regulated by bombesin and DHT as well as two additional sets of 62 and >100 genes specific for each agonist, respectively (Fig. 3B; Supplementary Tables S1-3). Of note, >53.7% of bombesin-regulated genes (i.e., 72 of 134) comprised the common set of bombesin/ARGs and 41% of those (30 of 72) have been previously identified as ARGs (Supplementary Table S1). Many of these genes can be classified as transcription factors and signal transduction proteins, suggesting that bombesin-activated AR may have a similar effect on cellular regulation as DHT-bound AR and may shed light on the processes critical for cell survival during androgen ablation crisis.

Of the remaining bombesin-regulated genes, we were intrigued to find that ~61% of the genes (38 of 62) were differentially regulated

To confirm the microarray results, we did reverse transcription-PCR analysis on several bombesin/ARs (Fig. 3C). This validated the up-regulation of 10 genes, including 7 known ARs (*KLK2*, *KLK3*, *KLK4*, *NKX3.1*, *TMEPAI*, *TMPRSS2*, and *SARG*). Comparison of band intensities showed that seven of the genes had similar levels of induction, whereas three were more strongly induced by either DHT (i.e., *KLK2* and *GREB1*) or bombesin (i.e., *KLK4*). Three of the genes we tested were differentially regulated: *CAMKK2* was induced by DHT but repressed by bombesin and *MYC* and *GRB10* were induced by bombesin but repressed by DHT. Although a putative ARE site has been identified in *CAMKK2* (28), an ARE site in either *MYC* or *GRB10* has not been reported and may be secondary targets of AR. Alternatively, they could be targets of signals that are differentially regulated by bombesin and androgen (see below). These results together support the idea that bombesin-activated AR had both similar and unique transcriptional activity compared with that of DHT-activated AR.

Bombesin-activated AR is preferentially recruited to the proximal promoter of prostate-specific antigen. The prostate-specific antigen (PSA) promoter has been well characterized (15), and AREs to which AR is recruited on ligand binding have been identified. We examined the ability of bombesin to recruit AR to the PSA promoter compared with DHT using ChIP. LNCaP cells were treated with either bombesin or DHT and analyzed for AR recruitment by ChIP assay. We found that bombesin could recruit AR to the proximal region of the PSA promoter albeit not as strongly as DHT (Fig. 4A). Unexpectedly, bombesin did not induce

AR recruitment to the enhancer ARE and apparently reduced constitutive AR occupancy to that region. We speculated that these results may translate to AR activation as well and tested this theory using luciferase reporter assays. LNCaP cells were transfected with a reporter construct containing the luciferase gene driven by either the PSA upstream sequences containing the enhancer element and proximal promoter (PSA6.0-Luc), the proximal promoter sequences (ARE-I/II-Luc), or the enhancer element (ARE-III-Luc). As expected, we found that bombesin could induce the activity of the reporter containing the proximal ARE regions (Fig. 4B). In contrast, the reporter containing only the enhancer region was not stimulated by bombesin and actually seemed to reduce the level of basal activity. Both bombesin and DHT stimulated activity of the full-length PSA promoter; however, only DHT seems to fully use the enhancer region, resulting in a 2-fold increase in activity compared with bombesin. These results suggest that bombesin-activated AR binds preferentially to particular AREs and provide the first indication that bombesin-activated AR may be conformationally different than androgen-bound receptor.

Bombesin-induced recruitment of a distinct cofactor complex to the PSA proximal promoter. If the conformation of bombesin-activated AR and androgen-bound AR is different, we reasoned that this may lead to a disparity in their cofactor assembly. Using ChIP, we assayed for the p160/SRC family of coactivators (SRC-1, ACTR, and TIF-2) as well as CBP and Pol II recruitment to the proximal PSA promoter. Additionally, we looked for *MYC* recruitment because we found *MYC* to be up-regulated

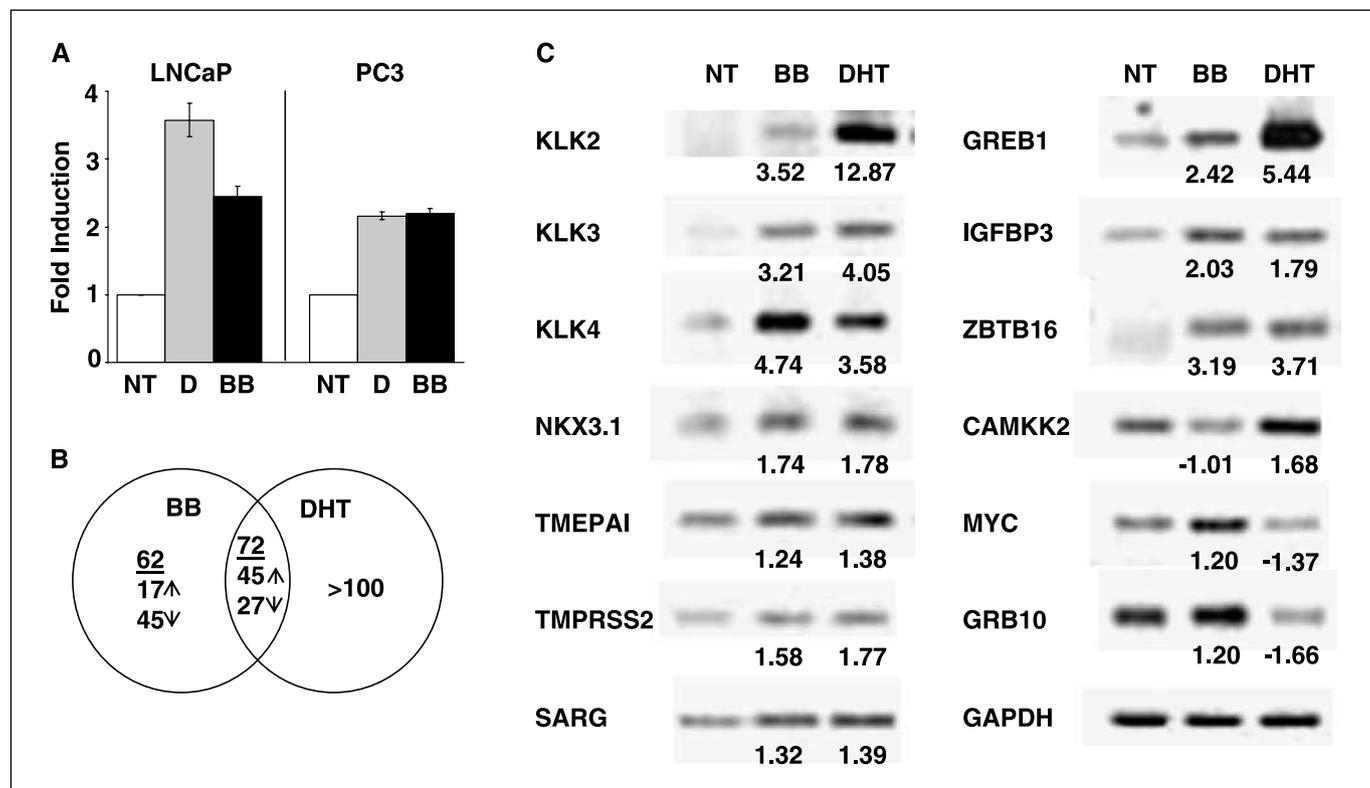


Figure 3. Bombesin activates AR and AR-responsive genes. *A*, androgen-deprived LNCaP or PC3 cells were transfected with ARE-Luc reporter plasmid or cotransfected with ARE-Luc reporter and pSG5-AR, respectively, under serum-free conditions for 24 hours followed by treatment with 100 nmol/L bombesin, 10 nmol/L DHT (*D*), or vehicle (*NT*) for 48 hours. Cell lysates were assayed for luciferase activity, and fold induction was calculated against control samples. *B*, microarray analyses of LNCaP cells treated for 6 hours with either 100 nmol/L bombesin or 10 nmol/L DHT were compared to identify commonly and uniquely modulated genes. *C*, androgen-deprived, serum-starved LNCaP cells were treated with 100 nmol/L bombesin, 10 nmol/L DHT, or vehicle for 6 hours. RNA was isolated using Trizol reagent, cDNA was synthesized, and PCR was done using primers for the indicated gene.

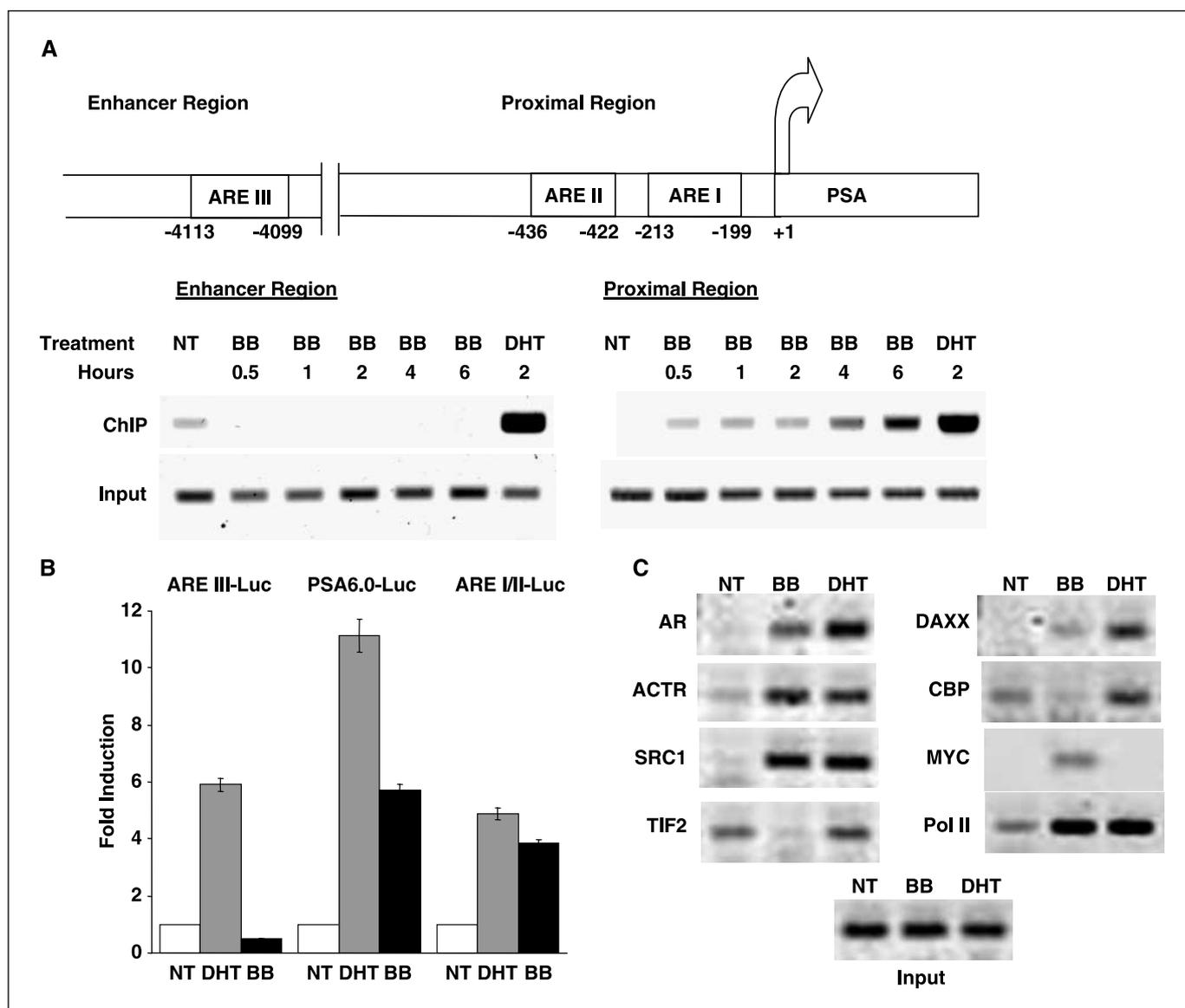


Figure 4. Bombesin-activated AR has unique conformational specificity and activity. *A*, androgen-deprived, serum-starved LNCaP cells were treated with 100 nmol/L bombesin, 10 nmol/L DHT, or vehicle for the indicated times and ChIP assay was done using α -AR (PG-21) antibody to immunoprecipitate protein-DNA complexes. The regions amplified are designated on the schematic of the PSA promoter. *B*, androgen-deprived LNCaP cells were transfected with pGL3-ARE-III-Luc, pGL3-PSA6.0-Luc or pGL3-ARE-I/II-Luc as indicated under serum-free conditions for 24 hours. Cells were then treated with 100 nmol/L bombesin, 10 nmol/L DHT, or vehicle for 48 hours, and cell lysates were assayed for luciferase activity. *C*, LNCaP cells were processed as in (*A*). In addition, rabbit α -ACTR (H-270), α -SRC-1 (M-341), α -TIF-2 (GRIP-1 M-343), α -DAXX (M-112), α -CBP (A-22), α -MYC (N-262), and α -Pol II (N-20) antibodies from Santa Cruz Biotechnology were used to immunoprecipitate complexes as specified.

by bombesin (Fig. 3C) and DAXX recruitment, which we recently showed that acts as a SUMO-dependent negative regulator of AR activity (29). We found that SRC-1, ACTR, and Pol II were recruited to the proximal region of PSA but could not detect any recruitment of TIF-2 or CBP (Fig. 4C). Interestingly, we found that MYC was recruited in response to bombesin treatment but not DHT, revealing a divergence in the transcriptional complex. Considering that MYC was activated by bombesin, we compared our microarray data with those on the Myc Cancer Gene database (30) and found that 28 genes were also known MYC targets, suggesting that MYC is an important mediator of bombesin action. Conversely, DAXX was recruited by DHT but only marginally recruited by bombesin. These results imply that bombesin-activated AR has similar yet distinct ability to recruit cofactors to the transcriptional complex

compared with DHT-activated AR. These differences may contribute to the differences in genes induced in response to bombesin compared with those induced in response to DHT.

Role of c-Src in bombesin-induced AR activation. In our previous report, we showed that bombesin induced Src kinase phosphorylation (6) and postulated that Src may be involved in bombesin-induced AR activation. We verified that bombesin induced Src activation in both LNCaP (Fig. 5A) and PC3 (data not shown) cells and that this was sensitive to the Src inhibitor PP2 (Fig. 5A). We next tested the effect of Src inhibition on LNCaP proliferation. LNCaP cells were pretreated with PP2 or Casodex before treatment with either bombesin or DHT, and cell growth was assayed. As shown in Fig. 5B, Src inhibition by PP2 had little effect on DHT-treated cells but could inhibit proliferation

transactivation but Casodex did not, whereas PP2 had no effect on DHT-induced AR activation.

We also examined the localization of AR induced by bombesin or DHT in LNCaP and PC3 cells pretreated with either PP2 or Casodex. In both cell lines, we found that PP2 could inhibit the translocation of AR induced by bombesin but not DHT, whereas pretreatment with Casodex did not prevent translocation induced by either stimuli (Fig. 2B and C, 4-7). We confirmed that inhibition of localization was Src selective by pretreating LNCaP cells with another Src inhibitor, SU6656 (31), before stimulation and again found that only in bombesin-treated cells could SU6656 inhibit AR translocation (Fig. 2B, 8 and 9). This suggests that Src activation leads to the modification of AR or an AR-associated protein(s) that allows its migration into the nucleus during nonsteroidal activation.

Finally, we analyzed the effect of PP2 on AR and cofactor recruitment to the PSA promoter by ChIP assay (Fig. 5D). We found that PP2 prevented the recruitment of AR, all the cofactors tested, and Pol II to the PSA promoter in bombesin-treated cells but not in DHT-treated cells. It has been reported that Casodex does not prevent recruitment of AR to the PSA promoter but rather prevents the recruitment of coactivators to inhibit AR action (32). Indeed, Casodex inhibited the recruitment of the ACTR, SRC-1, and Pol II to the promoter. Interestingly, Casodex alone does not inhibit the recruitment of MYC in bombesin-treated cells, whereas addition of PP2 does, suggesting that the recruitment of MYC to the PSA proximal promoter is Src dependent but AR independent (see Discussion).

GRP induces LNCaP proliferation, AR activation, and AR translocation. Bombesin has high homology to GRP, its human homologue, and has been shown to bind to human GRP receptors in PC3 cells (33). Still, we wanted to confirm that the results produced by bombesin could be repeated using GRP. We tested for proliferation and found that, like bombesin, GRP could replace androgen to support growth of LNCaP cells (Fig. 5B). This effect could be inhibited by both PP2 and Casodex. We next examined transcriptional activation of AR and found that GRP-induced activation of AR mirrored that of bombesin. GRP induction of the PSA6.0-Luc and ARE-I/II-Luc reporters could be inhibited by PP2 and Casodex, whereas repression of the ARE-III-Luc reporter was alleviated by PP2 but not by Casodex (Fig. 5C). Finally, we looked at cellular location of AR and found that GRP induced translocation of AR, which could be inhibited by PP2 (Fig. 2B, 10 and 11) but not by Casodex (data not shown). These data show that the AR-associated biological and signaling properties of bombesin and GRP are indeed conserved.

Discussion

Collectively, our data show that bombesin-induced AR activation proceeds via a mechanism that incorporates components used by conventional DHT-induced signaling and a novel, distinctive pathway dependent on GPCR-Src cross-talk, which has corresponding effects on the assembly of AR transcriptional complexes and gene expression patterns. We were able to show that bombesin action is not only a recapitulation of DHT mechanism but has unique differences in that bombesin-activated AR is recruited to specific ARE sites, assembles a divergent transcriptional complex, and regulates some but not all of the genes modulated by androgen. The most striking distinction is that bombesin-induced AR activation is mediated through Src kinase, suggesting that AR, or an associated protein, is phosphorylated in response to

bombesin. A recent report¹ showed that AR could be tyrosine phosphorylated directly by Src kinase, leading to nuclear translocation and enhanced AR activity. These data agree with our findings showing that Src is involved in bombesin-induced AR translocation and activation; however, we have not detected tyrosine-phosphorylated AR in bombesin-treated LNCaP cells (data not shown) and reflect that there are likely further downstream events that are involved. Active AR is serine phosphorylated (1), and phosphorylation of Ser⁶⁵⁰ is significant in AR transcriptional activity. Because ER can be phosphorylated by MAPK at Ser¹¹⁸ leading to ligand-independent activation of ER (8), it was proposed that AR may be similarly activated, although an analogous site on AR has not been found. We are able to show by *in vitro* kinase assay that bombesin-activated extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase can phosphorylate AR (data not shown); however, bombesin is still able to activate AR mutants that contain point mutations at known or putative phosphorylation sites (data not shown). This suggests that if AR is modified there could be unique residues on AR that are phosphorylated and/or unique serine/threonine kinases involved in the modification of AR. Novel kinases, such as CAK and PAK6, have been shown to associate with AR, but AR does not seem to be a substrate of these kinases (34, 35).

We have shown that bombesin-induced cofactor recruitment has a distinct pattern from DHT-induced assembly and is dependent on Src activation. It is reasonable therefore to postulate that kinase modification of cofactors instead of, or in addition to, AR could contribute to AR activation. In LNCaP cells, SRC-1 phosphorylated by MAPK led to ligand-independent activation of AR (36) and multiple kinases are reported to phosphorylate ACTR, which is required for both ER and AR coactivation (37). MAPK phosphorylation of ACTR has also been shown to facilitate p300 recruitment and histone acetyltransferase (HAT) activity in breast cancer (1). Bombesin-induced kinase activation could potentially lead to some of these modifications. A recent report showing that bombesin could activate p300 HAT activity leading to AR transactivation in prostate cells supports this theory (38).

Kinase involvement in the AR activation seems to be an early point of control in the mechanism, as loss of Src activation prevents AR translocation. One possibility is that Src is acting on another factor that can aid in the movement of AR into the nucleus, such as chaperone proteins. Classically, chaperone proteins are thought to be involved in the maintenance of AR in a partially unfolded ligand-unbound state to prevent aggregation of the protein. However, recent studies implicate chaperones in downstream AR signaling events, including translocation and transactivation of AR (39). It is conceivable that Src signaling could lead to the modification and/or recruitment of these factors to facilitate AR action. Indeed, heat shock proteins (HSP) are associated with Src and other downstream kinases (40). In particular, HSP70 proteins and cochaperones, such as HSP40 proteins, have been shown to aid in AR translocation and activation (41). Our microarray analysis showed up-regulation of HSP70 and HSP40 by both bombesin and DHT (HSPA1A, HSPA1B, DNAJB1, and DNAJB9) and by bombesin alone (DNAJA1). The combined effect of the modification and up-regulation of these chaperone proteins may assist in nonsteroidal activation of AR and ARGs.

¹ Z. Guo et al., abstract 2929, 97th AACR Annual Meeting 2006, Washington, DC.

We were intrigued to find that bombesin could not only induce expression of c-Myc but that c-Myc was also recruited to the PSA promoter in response to bombesin but not DHT. This is consistent with the observation that c-Myc is transcriptionally activated by Src kinase signaling (42). Recent publications have illustrated the ability of other transcription factors, such as FoxA1 (43) and c-Myc (44), to cooperate in the activation of ER target genes. We scanned the PSA promoter for c-Myc consensus sequences, CACGTG, and found three potential sites, one of which is located at -356 to -361 between ARE-I and ARE-II of the proximal PSA promoter. This position is consistent with the region amplified in the ChIP assay, supporting our finding that c-Myc is recruited to the proximal promoter. One of the key findings of this report is that bombesin-activated AR binds preferentially to certain ARE sites. However, in light of our c-Myc data, it may be that, rather than sequence bias, bombesin-activated AR binds to weak AREs situated in proximity to binding sites for other transcription factors, which mediate an indirect association of the AR with the chromatin by anchoring the AR to the promoter in the absence of ligand. We found that ~21% of our microarray genes were also c-Myc target genes, including 19 ARGs, supporting our data that c-Myc may be involved in bombesin-induced activation of AR in LNCaP cells. In our ChIP data, we show that Src inhibition prevented c-Myc

recruitment to the PSA promoter. Because AR recruitment is also abrogated by Src inhibition, it is unclear whether one transcription factor is dependent on the other for DNA occupancy. It is likely that AR and c-Myc recruitment are independent events that converge in the nucleus because bombesin has been shown to induce c-Myc expression in PC3 cells, which are AR negative (45). Additionally, TIP60 and GCN5/PCAF have been shown to acetylate c-Myc, leading to enhanced protein stability (46), and bombesin induction of acetyltransferases (38) could play a role in prolonging c-Myc activity. Aside from enhancing AR activation, c-Myc previously has been shown to play a role in androgen-independent growth (47) and prostate cancer progression (27). Indeed, transgenic mice models overexpressing c-Myc in the mouse prostate consistently developed poorly differentiated and invasive adenocarcinoma (48), showing a central role for c-Myc in prostate carcinogenesis.

The observations that Src is activated by many growth factors (1, 49), such as EGF and IGF-I, and implicated in other tumorigenic processes in prostate cancer, including migration (7), invasion, and angiogenesis (50), which are essential in the development of metastatic lesions, make it a promising candidate for targeted therapy in prostate cancer treatment. Our results substantiate the theory that, under crisis conditions, such as during androgen

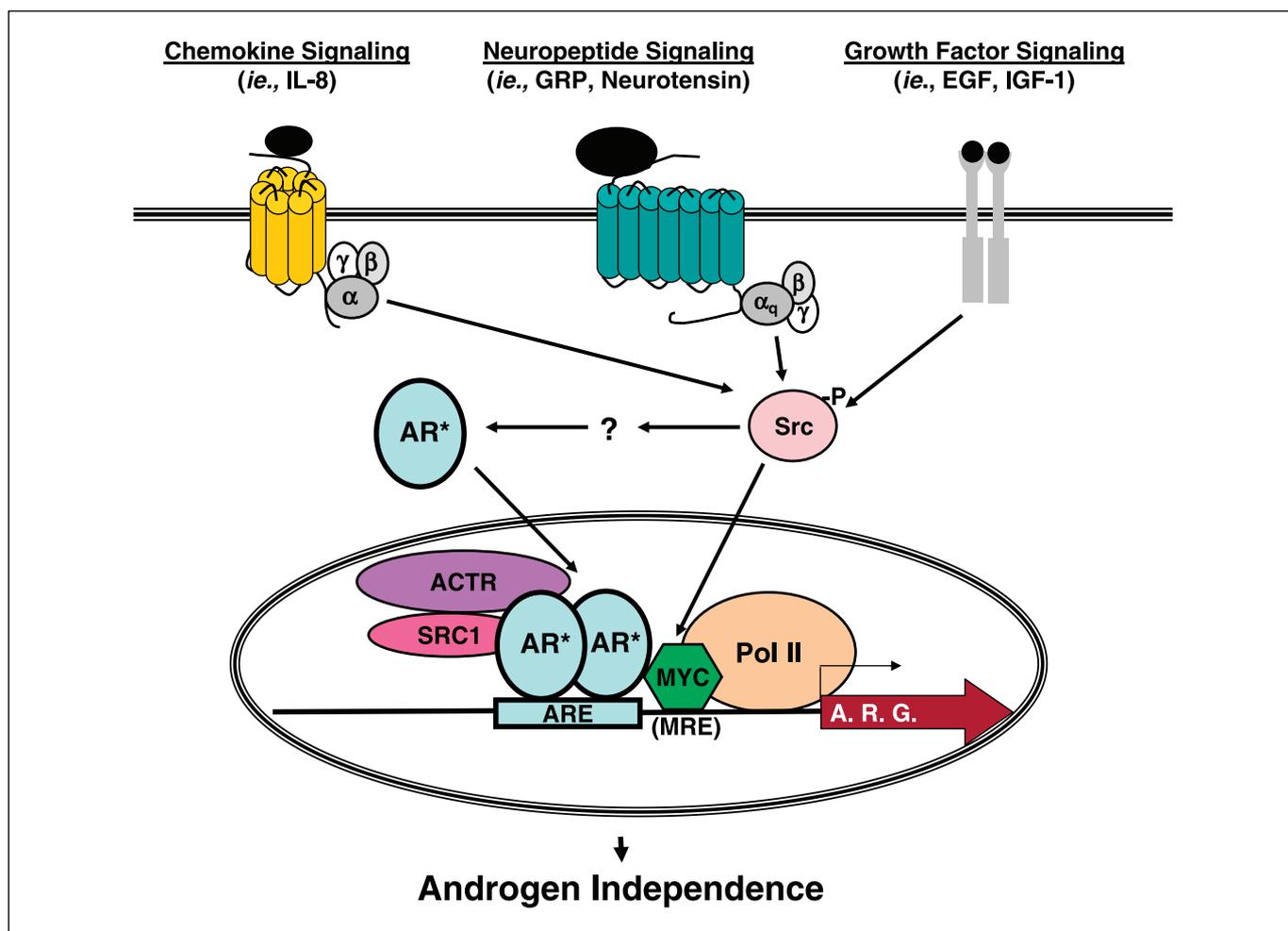


Figure 6. Model for transition to androgen independence in prostate cancer. Activation of Src kinase by multiple pathways can lead to the activation of c-Myc. Either directly or through other downstream signals, AR is modified (*) and teams up with c-Myc to induce ARGs required to sustain survival during androgen ablation crisis.

ablation treatment, activation of AR could be sustained through GPCR and growth factor signaling transmitted through Src kinase activation involving perhaps multiple ligands (Fig. 6). Targeting Src kinase in relapsed cancers could provide an earlier point of control for AR, may be beneficial as part of a combined treatment for better inhibition of AR (2), and presents an alternative treatment for unresponsive tumors that develop resistance to AR-targeted drugs, such as Casodex.

In this report, we have shown that bombesin can activate AR in a similar but distinctive mechanism compared with DHT-mediated activation and involves Src kinase. Although our studies were conducted in prostate cancer cells, evidence suggests that Src inhibition may be therapeutically beneficial. A recent report² showed that GRP-overexpressing LNCaP cells supported androgen- and anchorage-independent growth and that colony formation in soft agar could be inhibited by Src inhibition. *In vivo* studies found

LNCaP-GRP clones formed tumors in castrated nude mice and showed nuclear localization of AR and activation of PSA. Studies are under way to test the effects of Src inhibition on LNCaP-GRP tumors. The synergy that most likely exists between low androgen levels and other potential growth factors, such as GRP, in the human prostate during androgen ablation (2) has not been addressed in our current studies. However, having dissected the individual pathways of ligand versus nonligand activation of AR, we are now better able to examine how DHT and bombesin can act in concert to promote prostate cancer in our future studies.

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²J.C. Yang et al., abstract 3180, 96th AACR Annual Meeting 2005, Anaheim, California.

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