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RanBPM, a Nuclear Protein That Interacts with and Regulates Transcriptional Activity of Androgen Receptor and Glucocorticoid Receptor*

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The androgen receptor (AR) is a ligand-dependent transcription factor that has an essential role in the normal growth, development, and maintenance of the prostate gland. The AR is part of a large family of steroid receptors that also includes the glucocorticoid, progesterone, and mineralocorticoid receptors. Steroid receptor family members share significant homology at their DNA and ligand-binding domains. However, these receptors exhibit a high degree of sequence variability at their NH_2-terminal domain, which suggests the possibility of receptor-specific interactions with co-regulator proteins. Transcriptional co-regulators that interact with the AR may have a role in defining AR activity and may be involved in directing AR-specific responses. Here we have identified Ran-binding protein in the microtubule-organizing center (RanBPM) to be a novel AR-interacting protein by yeast two-hybrid assay and have confirmed this interaction by glutathione *S*-transferase- and His-tagged pull-down assays. In addition, transient overexpression of RanBPM in prostate cancer cell lines resulted in enhanced AR activity in a ligand-dependent fashion. Glucocorticoid receptor activity was also enhanced when RanBPM was overexpressed, whereas estrogen receptor activity remained unchanged. These data demonstrate that RanBPM interacts with steroid receptors to selectively modify their activity.

The androgen receptor (AR)[1] is a ligand-dependent transcription factor that belongs to a family of steroid receptors along with the glucocorticoid (GR), progesterone, and mineralocorticoid receptors. These steroid receptors share similar domain structures and mechanism of action. Steroid receptors including the AR have three functional domains: a COOH-terminal ligand-binding domain (LBD), a central DNA-binding domain (DBD), and an NH_2-terminal domain (1). In the absence of androgens, the AR is localized to the cytoplasm in an inactive complex that includes heat shock proteins (HSP). Upon binding to its cognate ligand, the AR undergoes a conformational change that results in a more compact and stable form of the AR. The activated AR dissociates from HSPs and translocates to the nucleus where it interacts with consensus DNA sequences as a homodimer to influence transcription of downstream genes (2). The estrogen receptor (ER) belongs to a different steroid receptor subfamily because it resides predominantly in the nucleus, even in its unliganded form, and does not require translocation across the nuclear membrane following activation (3). There are two major transactivation regions in the AR. 1) The activation function-1 (AF-1) domain is found at the NH_2 terminus. 2) AF-2 is located in the LBD. AF-2 is a weak transactivator that is dependent on the presence of androgens for its activation. AF-1, on the other hand, is capable of ligand-independent transactivation, and fragments of the AR that contain AF-1 show high levels of transcriptional activity when ectopically expressed in cell lines that are devoid of endogenous AR (4, 5).

Upon DNA binding, AR recruits components of the basal transcriptional machinery and influences either the up-regulation or down-regulation of gene expression. The exact mechanism of AR-specific gene expression is not fully understood. Each steroid receptor regulates unique sets of genes. However, *in vitro* assays have shown that these receptors recognize similar DNA sequences known as steroid response elements. These elements are comprised of a palindrome that contains two half-sites based on the 5′-TGTTCT-3′ motif that are separated by a three nucleotide spacer (6). Detailed analysis has demonstrated that both GR and AR bind with highest affinity to a steroid response element that has an imperfect palindrome, 5′-GGTACAnnnTGTTCT-3′ (7). The quandary is that although activated steroid receptors bind to highly homologous response elements on DNA, they still demonstrate an ability to regulate the expression of unique gene sets.

There are several mechanisms by which receptors can specifically regulate gene expression. One mechanism suggests that co-regulatory proteins interact with steroid receptors to direct their activity. The search for AR-specific co-regulatory molecules has led to the identification of several AR-interacting proteins. Early studies identified ARA70/ELE1 as a ligand-de-

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[1] The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; LBD, ligand-binding domain; DBD, DNA-binding domain; ER, estrogen receptor; AF-1, activation function-1; CREB, cAMP-response element-binding protein; CMV, cytomegalovirus; GST, glutathione *S*-transferase; FBS, fetal bovine serum; Luc, luciferase; TBS, Tris-buffered saline; ARR, androgen response regions; PSA, prostate-specific antigen; PB, probasin; TK, thymidine kinase; Dex, dexamethasone; ERK, extracellular signal-regulated kinase; RanBPM, Ran-binding protein in the microtubule-organizing center; SPRY, repeats in splA and RyR.

pendent co-activator of AR (8). Subsequently, CREB-binding protein/p300, which has histone acetyltransferase activity, was also shown to interact with AR and enhance receptor activity in prostate cells (9). More recent AR-interacting proteins that have been identified include β-catenin (10), caveolin (11), BAG-1L (12), SMAD3 (13), cyclin D1 (14), and several others. These proteins have been shown to either positively (caveolin and BAG-1L) or negatively (SMAD3 and cyclin D1) affect AR transactivation.

Identification of proteins that specifically interact with the AR has been a challenge because the AR and other steroid receptors share a high degree of sequence homology at their DBD and LBD. The DBD of human AR shares as much as 80% homology with that of progesterone receptor and over 70% with GR (15). The LBD of the steroid receptors are highly homologous as well with up to 55% similarity at the amino acid level. Therefore, many co-regulatory proteins that interact with the AR at the LBD and DBD are promiscuous in their ability to interact with and influence activity of other steroid receptors. A report by Alen *et al.* (16) has demonstrated that ARA70 is not specific to the AR and that this protein interacts with the ER and GR as well. Likewise, the steroid receptor co-activator-1/NCoA1, which is the founding member of the p160 family of transcriptional co-activators, interacts indiscriminately with the LBD of steroid receptors to enhance activity (17). Other members of the p160 family such as TIF-2/GRIP-1 enhance AR, GR and ER activity alike (18).

Nevertheless, steroid receptor family members show the greatest degree of sequence variability at the NH_2-terminal domain (<15%). Little is known regarding the role of the NH_2-terminal domain in AR transactivation. Therefore, we used an NH_2-terminal fragment of the AR, which is devoid of transcriptional activity ($AR_{1–232}$), as bait in a yeast two-hybrid assay, and RanBPM was identified as an AR-interacting protein. Although RanBPM was initially described as a 55-kDa protein (BPM55), a subsequent report has shown it to be a 90-kDa protein (BPM90) (19, 20). Here we demonstrate that the larger form of RanBPM, BPM90, is able to bind to multiple domains of the AR and that this interaction occurs *in vivo*. Furthermore, overexpression of RanBPM in prostate cancer cell lines shows that RanBPM can enhance AR transactivation. This property of RanBPM does not appear to be exclusive to the AR because BPM90 also enhances GR activity, although neither ER-α nor ER-β activity is affected. These experiments clearly demonstrate that RanBPM is capable of interacting with and modifying the activity of selective steroid receptors.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—A 696-bp fragment coding for the first 232 amino acids of the human AR ($AR_{1–232}$) was cloned into the *Bam*HI site of the pGBT9 vector for expression as a fusion protein with the GAL4 DBD (Clontech). A cDNA library made from normal human prostate tissue that was fused to the transactivation domain of GAL4 in the pACT2 expression vector was used for screening (Clontech). Expression plasmids were transformed into the Y190 yeast strain, and transformants were selected on SD minimal medium lacking tryptophan, leucine, and histidine. Clones that grew on minimal medium agar plates were subjected to β-galactosidase assays by colony filter-lift according to the manufacturer's instructions. Clones that tested positive for β-galactosidase were sequenced using Big Dye Terminator cycle sequencing reactions (Applied Biosystems) and were then compared with known sequences available in GenBank™.

Plasmid Construction—The full-length RanBPM expression vector, pcDEBΔ-BPM90, and empty vector, pcDEBΔ, were provided by Dr. H. Nishitani (Kyushu University) (20). The longest RanBPM library clone that was isolated in the yeast two-hybrid assay contained a 2152-bp fragment that coded for amino acids 148–729 of BPM90 and the 3′-untranslated region. This fragment of BPM90, herein referred to as BPM_L, was cloned into the pRC/cytomegalovirus (CMV) mammalian expression vector (Invitrogen) in which transcription is driven by the CMV promoter. BPM_L was cloned in-frame with an upstream ATG for translation initiation and a Kozak sequence to enhance translation efficiency.

The NH_2-terminal region of the human AR spanning the NH_2-terminal domain and DBD ($hAR_{1–646}$) was generated by PCR and cloned into the multiple cloning site of pRC/CMV for expression in mammalian cells. The full-length rat AR cDNA was expressed from the pRC/CMV mammalian expression plasmid, $pCMV/AR_6$ (21). The rat glucocorticoid receptor was expressed from the pGR mammalian expression vector as described elsewhere (22). The human ER-α expression vector (pSVMT:wER) has also been described previously (23). The pcDNA4/HisMax-hERβ1 vector (a gift from Dr. L. Murphy, University of Manitoba) was used for the expression of human ER-β in mammalian cells.

Northern Blot Analysis—Multiple tissue Northern blots (Clontech) were probed with [^{32}P]dCTP-labeled RanBPM cDNA. Poly(A) RNA was prepared from the LNCaP, PC3, MCF7, and HeLa cell lines using an Oligotex mRNA kit (Qiagen). For the Northern blot, 5 μg of poly(A) RNA were separated by electrophoresis on a 1% agarose gel with 30% formaldehyde and transferred to a Biodyne B nylon membrane (Pall Corporation) by capillary action in 10 mM NaOH. The membrane was hybridized with a [^{32}P]dCTP-labeled 1100-bp cDNA fragment coding for RanBPM. Glyceraldehyde-3-phosphate dehydrogenase was used to normalize the loading of poly(A) RNA.

GST and His-tag Pull-down Assay—Various NH_2-terminal and DBD fragments of the human AR ($AR_{1–232}$, $AR_{1–559}$, and $AR_{559–646}$) and a COOH-terminal fragment of rat AR ($AR_{DBD/LBD}$) were cloned into the pGEX vector (Amersham Biosciences) for expression as GST fusion proteins. GST fusion proteins were expressed in the BL21 *Escherichia coli* strain and purified as described previously (5). Radiolabeled RanBPM protein was prepared from the pRC/CMV-BPM_L vector using the Quick Coupled T7 TnT *in vitro* transcription/translation kit (Promega Corporation) in the presence of [^{35}S]Met. Equimolar amounts of GST-AR fusion protein coupled to glutathione-agarose beads were incubated with radiolabeled RanBPM at 4 °C for 2 h in binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% Nonidet P-40). Beads were washed four times with binding buffer, and bound proteins were eluted into protein sample buffer (2% SDS, 5% β-mercaptoethanol) for analysis by SDS-PAGE followed by autoradiography.

A fragment spanning the NH_2-terminal domain and DNA-binding domain of the AR ($AR_{1–646}$) was cloned into the pTrcHisC vector (Invitrogen) for expression with an NH_2-terminal His tag, which consists of six histidine residues in tandem. His-tagged proteins were expressed in bacteria and purified using the nickel-nitrilotriacetic acid-agarose column according to the manufacturer's protocol (Qiagen). [^{35}S]Met-RanBPM fragments were incubated with His-$AR_{1–646}$ at 4 °C for 4 h in binding buffer (see above). His-$AR_{1–646}$ was immunoprecipitated using an anti-His antibody (Qiagen) as described below.

Cell Culture and Transfection—PC3, HeLa, and MCF7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% fetal bovine serum (FBS) (Invitrogen) at 37 °C in 5% CO_2. The LNCaP prostate carcinoma cell line was cultured in RPMI 1640 medium containing 5% FBS. For transient transfection, 3×10^5 cells were seeded in six-well plates and were transfected the following day using Lipofectin reagent (Invitrogen) as described previously (24). Transfection occurred for 16 h at 37 °C. Following transfection, cells were re-fed with fresh medium containing 5% dextran-coated charcoal-stripped FBS ± 1 nM R1881, 10 nM dexamethasone (Dex), 10 nM E_2 or vehicle alone and incubated at 37 °C for an additional 24 h. After induction with hormone, cells were harvested and lysed in passive lysis buffer (Promega Corporation) for luciferase assay and for Western blot analysis.

Immunoprecipitation and Western Blotting—LNCaP cells were grown to 80% confluency in RPMI 1640 medium + 5% FBS. Cells were then cultured in RPMI 1640 medium containing 5% dextran-coated charcoal-stripped FBS for 16 h at 37 °C. The following day, cells were induced in the presence or absence of 10 nM R1881 for 4 h at 37 °C before scraping and lysis in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Whole cell extracts (1 mg of protein) were incubated with a polyclonal rabbit anti-AR antibody or with normal rabbit IgG as a negative control (Santa Cruz Biotechnology, Inc.). Immunocomplexes were pulled down using protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) and washed four times with radioimmune precipitation buffer. After the final wash, proteins were solubilized in SDS sample buffer and analyzed by Western blot.

Western blots were carried out as described previously (21). Protein samples were resolved on a polyacrylamide gel and transferred to a

polyvinylidene difluoride membrane (Millipore). Membranes were blocked in TBS (20 mM Tris-Cl, pH 7.6, 137 mM NaCl) with 5% skim milk. Blots were incubated with appropriate primary antibody, diluted to 2 μg/ml in TBS + 5% milk for 4 h at room temperature, washed three times in TBS + 0.5% Tween 20, and then incubated for 45 min in horseradish peroxidase-conjugated secondary antibody (1:10,000) (Santa Cruz Biotechnology, Inc.). Blots were developed using the ECL chemiluminescence kit (Amersham Biosciences).

Transcription Assays—AR and GR constructs were co-transfected with the pARR3-tk-Luc reporter construct in which the promoter has three androgen response regions (ARRs) in tandem (5). In addition, the prostate-specific antigen (PSA) and probasin (PB) luciferase reporter constructs were used to determine transcriptional activity of the AR (4, 24). ER expression vectors were co-transfected with the pERE-Luc reporter plasmid as described previously. The pERE-Luc plasmid contains a single vitellogenin estrogen response element upstream of the thymidine kinase (TK) promoter.[2] Transfected cells were incubated in the presence or absence of hormone at 37 °C for 24 h prior to analysis. Transfection efficiency was normalized using the Renilla luciferase expression vector, pRL-TK (Promega Corp.). Firefly and Renilla luciferase activities were assayed with the Dual Luciferase assay kit (Promega Corp.). 20 μl of cell lysate were analyzed for luciferase activity using MicroLumiat*Plus* luminometer (EG&G Berthold).

RESULTS

RanBPM Interacts with the NH_2 Terminus of AR in Yeast—A fragment coding for the first 232 amino acids of the AR (AR_{1-232}) was used as bait in a yeast two-hybrid assay to screen a human prostate library for interacting proteins. Over 3×10^5 transformants were screened on selective media, and positive clones were identified by β-galactosidase assay. Sequence analysis of three independent positive clones revealed a gene that was highly homologous to a known human protein, RanBPM (GenBank™ accession number AB055311) (19). Both 55- and 90-kDa forms of RanBPM have been reported in the literature and are referred to as BPM55 and BPM90, respectively (19, 20). Sequences isolated from the three library clones isolated by the yeast two-hybrid assay had sequences upstream of the published BPM55 translation start codon (Fig. 1). The longest sequence of the three library clones (ARBP1) was 2346 bp in length and coded for a protein from amino acid 148 of BPM90 to the termination codon. This fragment is referred to as BPM_L.

RanBPM Interacts with AR in Vitro and in Vivo—To confirm the interaction between RanBPM and AR that was identified by yeast two-hybrid screening, various AR constructs were expressed as GST fusion proteins and coupled to glutathione-agarose beads for use in GST pull-down assays. The 2346-bp fragment from ARBP1 (BPM_L) was cloned into pRC/CMV to allow for *in vitro* transcription/translation from the T7 promoter. [^{35}S]Methionine-labeled BPM_L protein was allowed to interact with the GST-AR fusion proteins and analyzed by SDS-PAGE followed by autoradiography. As seen in Fig. 2*A*, RanBPM interacts with the 232-amino acid fragment that was originally used as bait in the yeast two-hybrid screen (AR_{1-232}) (Fig. 2*A*, *lanes 3* and *4*). Equimolar amounts of AR-GST fusion proteins were used to determine relative binding between RanBPM and various domains of AR, and the results show that RanBPM interacts most strongly with the DBD ($AR_{559-646}$) (Fig. 2*A*, *lane 2*). In addition, RanBPM interacts with the AR fragment that spans the DBD and LBD ($AR_{DBD/LBD}$) (Fig. 2, *lane 5*). However, presence of the LBD does not enhance interaction, which suggests that the LBD does not have additional domains for RanBPM binding.

To assess the relevance of this interaction *in vivo*, we performed a co-immunoprecipitation assay using LNCaP cell lysates. The LNCaP prostate carcinoma cell line expresses high levels of functional AR and displays androgen-dependent gene expression activity. Cells were treated for 4 h with 10 nM R1881 or with vehicle alone prior to preparation of protein lysates. Whole cell lysates were immunoprecipitated with normal rabbit IgG as a negative control (Fig. 2*B*, *lanes 3* and *5*) or with an antibody that recognizes the COOH terminus of the AR (*lanes 4* and *6*) (Santa Cruz Biotechnology, Inc.). Protein complexes were pulled down with protein A/G coupled to agarose beads. Proteins of the AR complex were resolved by SDS-PAGE prior to Western blotting with an anti-BPM90 antibody (provided by Dr. H. Nishitani). A 90-kDa protein that is seen in the input lanes (Fig. 2*B*, *lanes 1* and *2*) is BPM90. The results in Fig. 2*B* show that BPM90 interacts with AR specifically in the presence of hormone (*lanes 3–6*). We confirmed that AR was pulled-down in this assay by blotting the same membrane with an antibody that recognizes the NH_2 terminus of the AR (Affinity BioReagents) (Fig. 2*B*).

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  1 MSGQPPPPPP QQQQQQQQLS PPPPAALAPV SGVVLPAPPA VSAGSSPAGS
 51 PGGGAGGEGL GAAAAALLLH PPPPPPPATA APPPPPPPPP PPASAAAPAS
101 GPPAPPGLAA GPGPAGGAPT PALVAGSSAA APFPHGDSAL NEQEKELQRR
151 LKRLYPAVDE QETPLPRSWS PKDKFSYIGL SQNNLRVHYK GHGKTPKDAA
                                  *
201 SVRATHPIPA ACGIYYFEVK IVSKGRDGYM GIGLSAQGVN MNRLPGWDKH
251 SYGYHGDDGH SFCSSGTGQP YGPTFTTGDV IGCCVNLINN TCFYTKNGHS
301 LGIAFTDLPP NLYPTVGLQT PGEVVDANFG QHPFVFDIED YMREWRTKIQ
351 AQIDRFPIGD REGEWQTMIQ KMVSSYLVHH GYCATAEAFA RSTDQTVLEE
401 LASIKNRQRI QKLVLAGRMG EAIETTQQLY PSLLERNPNL LFTLKVRQFI
451 EMVNGTDSEV RCLGGRSPKS QDSYPVSPRP FSSPSMSPSH GMNIHNLASG
501 KGSTAHFSGF ESCSNGVISN KAHQSYCHSN KHQSSNLNVP ELNSINMSRS
551 QQVNNFTSND VDMETDHYSN GVGETSSNGF LNGSSKHDHE MEDCDTEMEV
601 DSSQLRRQLC GGSQAAIERM IHFGRELQAM SEQLRRDCGK NTANKKMLKD
651 AFSLLAYSDP WNSPVGNQLD PIQREPVCSA LNSAILETHN LPKQPPLALA
701 MGQATQCLGL MARSGIGSCA FATVEDYLH
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FIG. 1. **Amino acid sequence of BPM90 (GenBank™ accession number BAB62525).** AR_{1-232} was used as bait to screen a normal prostate cDNA library for interacting proteins. Three independent cDNA clones coded for RanBPM. The *underlined* portion shows the protein encoded by the longest cDNA sequence that was isolated (herein referred to as BPM_L). The *asterisk* denotes the translation start site of BPM55 (GenBank™ accession number BAA23216). *Shaded sequence* is the putative SPRY domain.

The NH_2 Terminus of RanBPM Is Essential For interaction with AR—RanBPM encodes a putative SPRY domain at the NH_2 terminus (Fig. 1) (19). This domain has been implicated in protein-protein interactions. To determine the importance of the SPRY domain, His-tag pull-down assays were carried out to map the interacting domains between RanBPM and the AR. Radiolabeled COOH-terminal truncations of RanBPM were generated by restriction digest of the pRC/CMV-BPM_L vector with *Eco*RI or *Nde*I followed by *in vitro* transcription/translation in the presence of [^{35}S]methionine. The truncated RanBPM proteins ($BPM_{148-408}$ and $BPM_{148-251}$) were incubated with purified recombinant His-tagged AR_{1-646}, which includes the NH_2-terminal domain and DBD. An antibody that recognizes the His-tag (Qiagen) was used to immunoprecipitate AR and its interacting proteins. After sufficient washing, samples were eluted into protein sample buffer, resolved by SDS-PAGE, and analyzed by autoradiography. Fig. 2*C*, *Input lanes*, show that radiolabeled proteins have molecular masses of 64, 28, and 11 kDa (BPM_L, $BPM_{147-408}$, and $BPM_{147-251}$, respectively). Both BPM_L and the truncated peptide generated by *Eco*RI digest, $BPM_{147-408}$, interacted with the AR (Fig. 2*C*, *top* and

[2] J. L. Ralph, M. C. Orgebin-Crist, J.-J. Lareyre, and C. C. Nelson, manuscript in preparation.

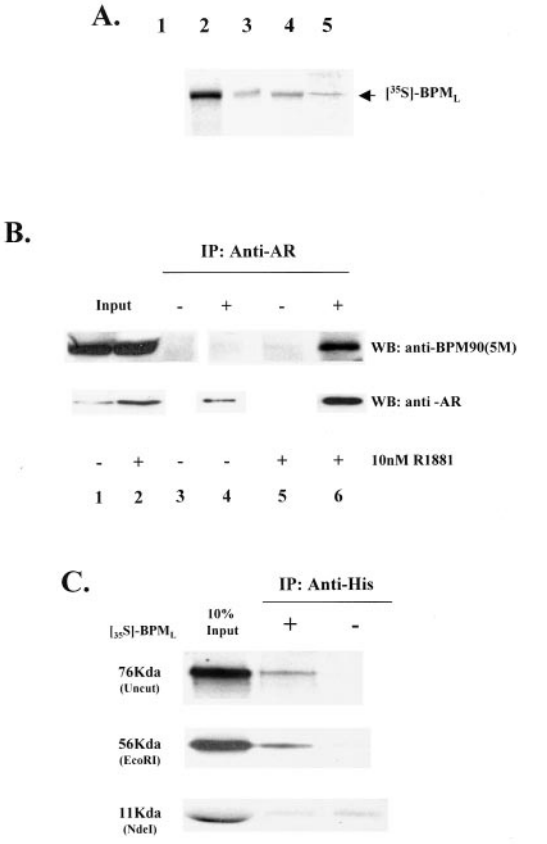

FIG. 2. **RanBPM interacts with the NH_2-terminal domain and DBD of the AR.** *A*, [^{35}S]Methionine-labeled BPM_L was allowed to interact with GST, GST-$AR_{559-646}$, GST-AR_{1-232}, GST-AR $_{1-559}$, and GST-$AR_{DBD/LDB}$ (*lanes 1–5*, respectively) coupled to glutathione-agarose beads at 4 °C overnight. Proteins were eluted from washed beads with protein sample buffer and resolved on an SDS-PAGE gel before analysis by autoradiography. *B*, RanBPM co-immunoprecipitates with AR. LNCaP cells were induced with (*lanes 2*, *5*, and *6*) or without (*lanes 1*, *3*, and *4*) 10 nM R1881 for 4 h prior to lysis in radioimmune precipitation buffer. Whole cell extracts were incubated with normal rabbit IgG (*lanes 3* and *5*) or an antibody to the COOH terminus of AR (*lanes 4* and *6*). Input protein lysates from LNCaP cells grown, − or + R1881, are shown in *lanes 1* and *2*, respectively. Proteins were pulled down using protein A/G coupled to agarose beads and resolved on an 8% SDS-PAGE gel prior to Western blot with an antibody that recognizes BPM90 (*top panel*) or the NH_2 terminus of AR (*bottom panel*). *C*, the pRC/CMV-BPM_L vector was used for *in vitro* transcription/translation in the presence of [^{35}S]methionine (*top panel*). Radiolabeled truncated fragments of BPM_L were generated by restriction digest of the expression vector with *Eco*RI ($BPM_{148-408}$; *middle panel*) or *Nde*I ($BPM_{148-251}$; *bottom panel*) followed by *in vitro* labeling. The fragments of RanBPM were incubated with His-AR_{1-646} for 4 h prior immunoprecipitation with an anti-His antibody (*lane 2*) or normal mouse IgG as a negative control (*lane 3*). 10% input of the labeled protein is seen in *lane 1*.

middle panels). However, $BPM_{147-251}$ in which the SPRY domain is disrupted was no longer able to interact with AR (Fig. 2*C*, *bottom panel*). This finding agrees with yeast two-hybrid results in which β-galactosidase activity and, therefore, interaction with AR_{1-232} are lost when an NH_2-terminal truncation of RanBPM, which does not have a complete SPRY domain, is used as bait (data not shown).

RanBPM Is Ubiquitously Expressed—To determine expression levels of RanBPM, a [^{32}P]cDNA probe was generated from the ARBP1 yeast two-hybrid library clone and hybridized to a multiple tissue Northern blot (Clontech). This Northern blot allows for analysis of RanBPM expression in several different tissue types. Fig. 3*A* shows that RanBPM is expressed as a 2.9-kb transcript in multiple different tissue types. High levels of RanBPM message are seen in the prostate and ovaries, and

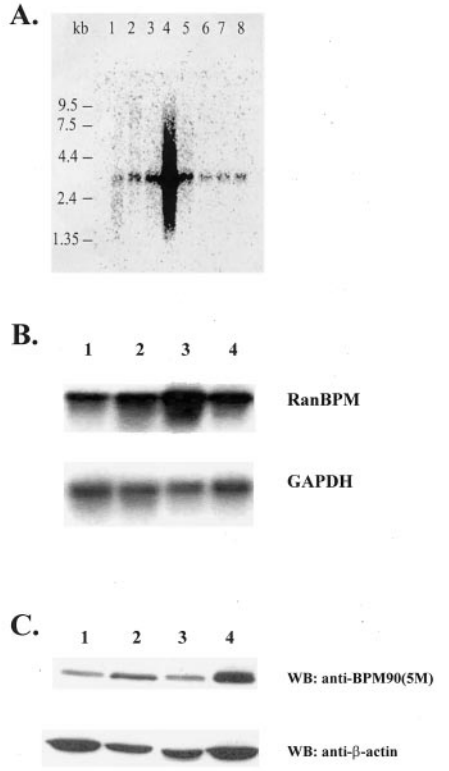

FIG. 3. **RanBPM is ubiquitously expressed.** *A*, A multiple tissue Northern blot (Clontech) was analyzed with a [^{32}P]cDNA probe coding for RanBPM to determine the size and expression profile of the RanBPM transcript. Tissue samples on the membrane included spleen (*lane 1*), thymus (*lane 2*), prostate (*lane 3*), testes (*lane 4*), ovaries (*lane 5*), small intestine (*lane 6*), colon (*lane 7*), and peripheral blood lymphocytes (*lane 8*). *B*, poly(A) RNA was isolated from four carcinoma cell lines for Northern blot analysis. 5 μg of mRNA from each cell line was run on a 1% agarose gel with 30% formaldehyde and transferred to a nylon membrane by capillary action. Samples include PC3 and LNCaP prostate cancer cell lines (*lanes 1* and *2*, respectively), the MCF7 breast carcinoma cell line (*lane 3*), and a cervical cancer cell line, HeLa (*lane 4*). The membrane was hybridized with a radiolabeled RanBPM cDNA probe (*top panel*). Loading efficiency was normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (*bottom panel*). *C*, RanBPM protein expression was assessed for the four cancer cell lines described above. Total protein (50 μg) was resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Samples include PC3, LNCaP, MCF7, and HeLa cell lines (*lanes 1–4*, respectively). An antibody specific for BPM90 (20) was used for Western blot analysis (*top panel*). Protein loading efficiency was normalized by β-actin (*bottom panel*).

highest levels of expression are observed in the testes (*lanes 3*, *5*, and *4*, respectively).

RanBPM expression levels were determined for various cancer cell lines by both Northern and Western blots (Fig. 3, *B* and *C*). Cell lines that were used included the ovarian carcinoma HeLa cell line from which BPM90 was first identified, two prostate cancer cell lines, PC3 and LNCaP, and the MCF7 breast cancer cell line. Both Northern and Western blots show that RanBPM is highly expressed in all of the cell lines. In Fig. 3*C*, the 90-kDa protein seen in *lanes 1–4* is BPM90. Although BPM90 is expressed in all of the cell lines tested, the highest levels were observed in HeLa cells (*lane 4*). PC3 and MCF7 cells expressed lower levels of BPM90 as compared with the LNCaP cell line (compare *lanes 1* and *3* with *lane 2*). The membrane was probed with an antibody for β-actin (Sigma) to demonstrate that loading efficiency was consistent in all lanes (Fig. 3*C*).

RanBPM Enhances the Activity of the Androgen Receptors—Our results have demonstrated that RanBPM interacts with

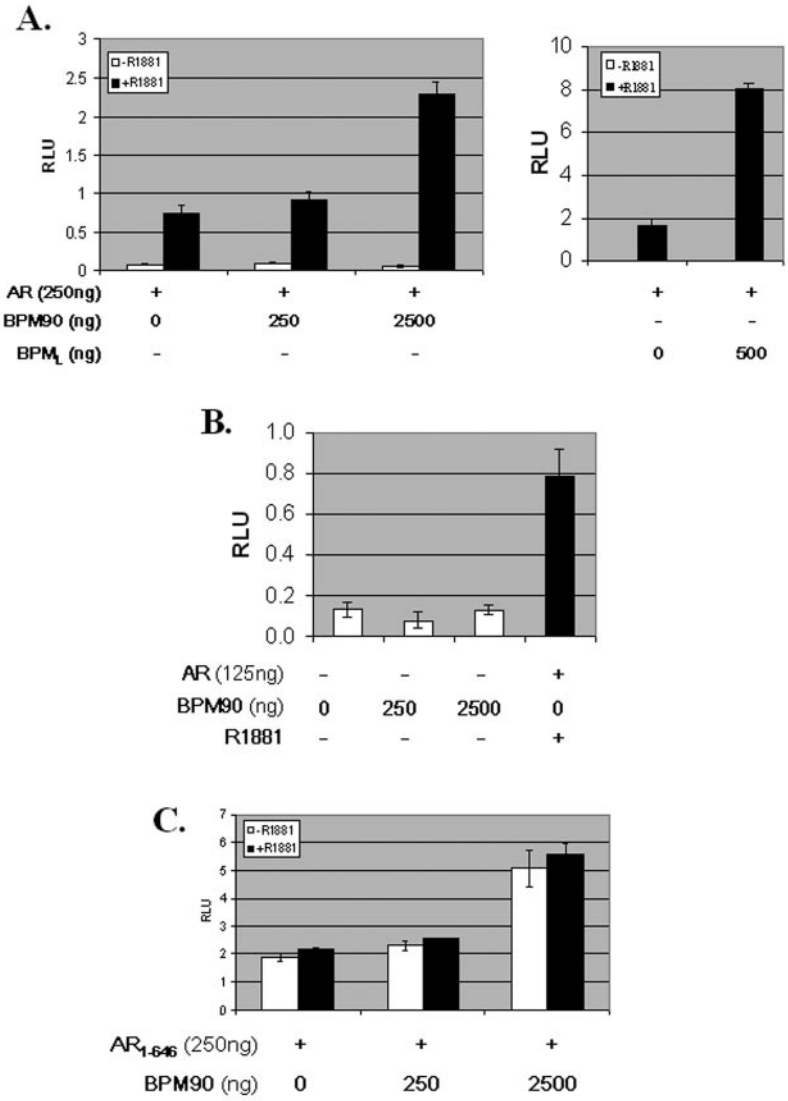

FIG. 4. **RanBPM enhances AR activity.** *A*, PC3 cells were transfected with full-length AR (250 ng/well), pARR3-tk-Luc (167 ng/well), and increasing amounts of pcDEBΔ-BPM90 or pRC/CMV-BPM$_L$ (0, 250, 500, or 2500 ng/well). DNA was kept constant at 3 μg/well by the addition of empty vector. Transfection was performed using Lipofectin reagent (Invitrogen) for 16 h at 37 °C. Cells were induced in the presence (■) or absence (□) of 1 nM R1881 for 24 h before harvesting for luciferase assay. *B*, RanBPM does not change AR activity in the absence of ligand. PC3 cells were transfected with AR (125 ng/well) and pARR3-tk-Luc (167 ng/well) and BPM90 at increasing ratios with AR (0:1, 2:1, 20:1) as described above. Transfected cells were maintained in Dulbecco's modified Eagle's medium + 5% stripped FBS without additional androgens (□) or with 1 nM R1881 (■) for 24 h prior to analysis. DNA was kept constant at 3 μg/well by the addition of empty vector. *C*, RanBPM can enhance the activity of a constitutively active fragment of the AR. AR$_{1-646}$ encodes the full NH$_2$-terminal domain and DBD of the AR and exhibits ligand-independent transactivation. PC3 cells were transfected with pRC/CMV-AR$_{1-646}$ (250 ng/well), pARR3-tk-Luc, and BPM90 (1, 250, or 2500 ng/well) as described above. Cells were induced for 24 h at 37 °C with 1 nM R1881 (■) or with vehicle alone (□). For all experiments, Western blot analysis was carried out on lysates to ensure that AR levels were constant (data not shown). Transfection efficiency was normalized with a Renilla luciferase expression vector (pRL-TK). Values are the average of triplicates. Each graph is representative of three independent experiments.

the androgen receptor at the NH$_2$ terminus and at the DNA-binding domain (Fig. 2*A*). To determine whether this interaction has a biological impact on AR activity, transcriptional assays were carried out using the PC3 cell line. PC3 cells represent a relatively undifferentiated stage of prostate cancer. These cells express very little to no androgen receptor and do not demonstrate androgen-regulated growth. Cells were transiently transfected with an AR expression vector (pCMV/AR$_6$), the pARR3-tk-Luc reporter plasmid, and increasing amounts of RanBPM (pcDEBΔ-BPM90) and then were induced in the presence or absence of 1 nM R1881 for 24 h prior to harvesting for luciferase assays (Fig. 4*A*). In the absence of hormone, negligible AR-mediated transcriptional activity was observed from the pARR3-tk-Luc reporter plasmid. An addition of hormone resulted in an ~9-fold induction of AR activity. In the presence of hormone, the overexpression of BPM90 resulted in an AR activity that was three times greater than in the absence of RanBPM. AR activity in the absence of hormone was unchanged by the addition of RanBPM, even with a 20-fold excess of BPM90 (Fig. 4*B*). Western blot analysis of cell lysates was carried out to ensure equivalent levels of AR expression in all samples (data not shown). Additional transactivation assays were carried out using an NH$_2$-terminal truncated form of RanBPM (BPM$_L$) in which expression is under the control of a CMV promoter (Fig. 4*A*). The presence of BPM$_L$ increases basal AR activity by 4-fold when androgens are present. BPM$_L$ did not affect the activity of AR in the absence of ligand when the AR was transcriptionally inactive.

Because RanBPM was identified by its interaction with the NH$_2$ terminus of the AR, similar transactivation experiments were carried out with a form of the AR that is deleted for the LBD, pRC/CMV-AR$_{1-646}$. This region of the AR has the AF-1 site and is capable of ligand-independent transcriptional activity. PC3 cells were transiently transfected with the truncated AR construct and treated with or without 1 nM R1881. AR$_{1-646}$ is capable of high levels of transcriptional activity both in the presence and absence of ligand (Fig. 4*C*). Increasing amounts of BPM90 resulted in increased AR$_{1-646}$ activity up to 2.5-times greater than basal levels. This increase in AR activity was independent of added hormone.

The ARR3 promoter of the reporter plasmid is a synthetic highly active promoter that has three ARRs in tandem. The PB and PSA promoters, however, may be more physiologically relevant. *In vivo*, the expression of the rat probasin gene is restricted to the prostate and is regulated by the AR (21, 25). PSA gene expression is also regulated by AR activity and is used as a molecular marker for prostate cancer progression (26). Luciferase reporter constructs under the control of the PB and PSA promoters were also used to determine the effect of BPM90 overexpression on AR activity (Fig. 5, *A* and *B*). PC3 cells transfected with pCMV/AR$_6$ and PB-Luc demonstrated a slight increase in ligand-dependent AR activity in the presence

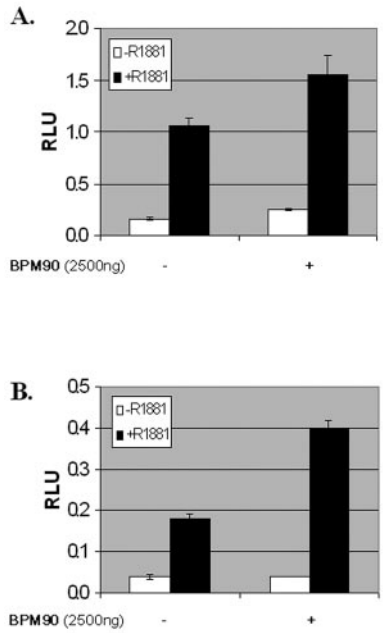

FIG. 5. **BPM90 enhances AR activity on multiple AR-regulated promoters.** PC3 cells were transfected as described previously with full-length AR, the pPB-Luc (*A*) or pPSA-Luc (*B*) reporter plasmids (167 ng/well) with the addition (+) or absence (−) of pcDEBΔ-BPM90 (2500 ng/well). Relative luciferase units were measured following a 24-h induction with 1 nM R1881 (■) or with vehicle alone (□). Transfection efficiency was normalized with pRL-TK. Values are the average of triplicates. Each graph is representative of three independent experiments.

of BPM90 (Fig. 5*A*). On the other hand, the overexpression of BPM90 resulted in a 2-fold increase in ligand-dependent AR transactivation from the PSA-Luc reporter construct (Fig. 5*B*). AR expression levels remained constant under the different treatments as determined by Western blot (data not shown).

RanBPM Specifically Regulates AR- and GR-mediated Transactivation—To determine whether RanBPM could enhance transactivation of AR alone or of other steroid receptors as well, transcription assays were carried out with GR, ER-α, and ER-β. PC3 cells were transfected with expression plasmids for GR, ER-α, or ER-β (250 ng/well) and with increasing amounts of BPM90 prior to induction with 10 nM Dex or 10 nM E_2, respectively (Fig. 6*A*). In PC3 cells, GR shows an 11-fold induction in the presence of 10 nM Dex (Fig. 6*A*, *lane 1*). This activity increases with the overexpression of BPM90 as seen in Fig. 6*A*, *lane 2*. High levels of BPM90 (2.5 μg/well) gave a 31-fold induction of GR activity in the presence of Dex (Fig. 6*A*, *lane 2*). Similar to the AR, GR activity was unchanged by high levels of BPM90 in the absence of ligand (data not shown).

In addition, estrogen receptor was ectopically expressed in PC3 cells by transient transfection of either the pSMVT:wtER or pcDNA4-hERβ1 expression vector (250 ng/well) (Fig. 6*A*, *lanes 3–6*). ER-α activity in PC3 cells showed a greater than 1.7-fold induction in the presence of ligand (10 nM E_2) (Fig. 6*A*, *lane 3*). This activity was relatively unchanged by the expression of RanBPM. Even when BPM90 was present at a 10:1 ratio with the receptor (2500 ng of BPM90/well), ER-α-mediated transactivation remained the same (*lane 4*). Similarly, ER-β showed a 2-fold induction in the presence of 10 nM E_2 regardless of the presence of BPM90 (Fig. 6*A*, *lanes 5* and *6*).

To assess the affect of RanBPM in alternate cell environments, prostate cancer LNCaP and breast carcinoma MCF7 cell lines were used (Fig. 6, *B* and *C*). Cells were transiently transfected with the expression vector for BPM90 or with empty vector pcDEBΔ and steroid receptor expression vectors (250 ng/well) to determine whether RanBPM overexpression could modify steroid receptor activity. In LNCaP cells, AR has very high levels of activity when induced with 1 nM R1881 for 24 h (~64-fold induction). AR activity was 2.8-times greater when high levels of BPM90 (2500 ng/well) were expressed in LNCaP cells as determined by luciferase assay (Fig. 6*B*). A similar enhancement of transcriptional activity, 2-fold increase in the presence of 10 nM Dex, was seen in LNCaPs that were co-transfected with GR and BPM90 (Fig. 6*B*). On the other hand, the difference in either ER-α or ER-β activity in MCF7 cells in the presence or absence of BPM90 was not statistically significant (Fig. 6*C*). These results demonstrate that RanBPM is able to modify AR and GR activity in at least two different prostate cancer cell lines but did not influence ER activity in either prostate or breast cancer cell lines.

DISCUSSION

The mechanism that dictates AR-specific transactivation is still unclear. Upon ligand binding, the AR translocates to the nucleus, binds to DNA, and recruits members of the basal transcription machinery. Unique protein-DNA interactions in the promoter/enhancer region of AR-regulated genes are one mechanism by which AR-specific transcriptional regulation may be attained. Recent studies by Reid *et al.* (27) show that the AR interacts with two different classes of androgen receptor response elements in the rat probasin promoter. The expression of the probasin gene in rats is highly restricted to the prostate and is regulated by AR. In addition to known interactions between the AR and conventional class I androgen receptor response elements, these studies have demonstrated novel interactions that occur with a class II androgen receptor response elements, which may add specificity to AR transactivation responses. Other studies have demonstrated that unique receptor-DNA interactions can occur for different steroid receptors as well (28, 29).

Co-regulatory molecules that interact with AR and the transcription apparatus to enhance or repress gene expression may also impart a degree of receptor-specific activity. Although several AR-interacting co-regulatory proteins have been identified, the majority of these proteins interacts with AR at the LBD and DBD, which are regions of high homology between steroid receptor family members. AR and progesterone receptor share up to 80% homology at the DBD, whereas AR and GR share ~55% homology at the LBD (15). As a result, several co-regulatory proteins such as steroid receptor co-activator-1, ARA70, CREB-binding protein/p300), and the SWI/SNF chromatin-remodeling proteins are not unique to the AR and have been shown to interact with other steroid receptors (reviewed in Ref. 30).

In this study, RanBPM was identified as an AR-binding protein. An NH_2-terminal fragment of the AR (AR_{1-232}) was used as bait in the yeast two-hybrid assay, and subsequent GST pull-down assays have confirmed the interaction (Figs. 1 and 2*A*). Traditionally, the NH_2-terminal domain of the AR is incompatible with yeast two-hybrid assays because of the ligand-independent transcriptional activity of the AF-1 domain (5). However, the AR fragment that was used as bait is truncated for AF-1 and is devoid of transactivation properties. Furthermore, AR_{1-232} is of interest because there are no homologous regions found in other steroid receptors.

RanBPM was originally identified by its interaction with Ran, a small Ras-like GTPase (19). Although RanBPM was initially identified as a 55-kDa protein (BPM55), a longer form has been reported more recently (20). The novel RanBPM protein, BPM90, is a 90-kDa protein that demonstrates nuclear and perinuclear localization in HeLa and KB cells lines. The function of BPM90 is still unclear, but it has recently been

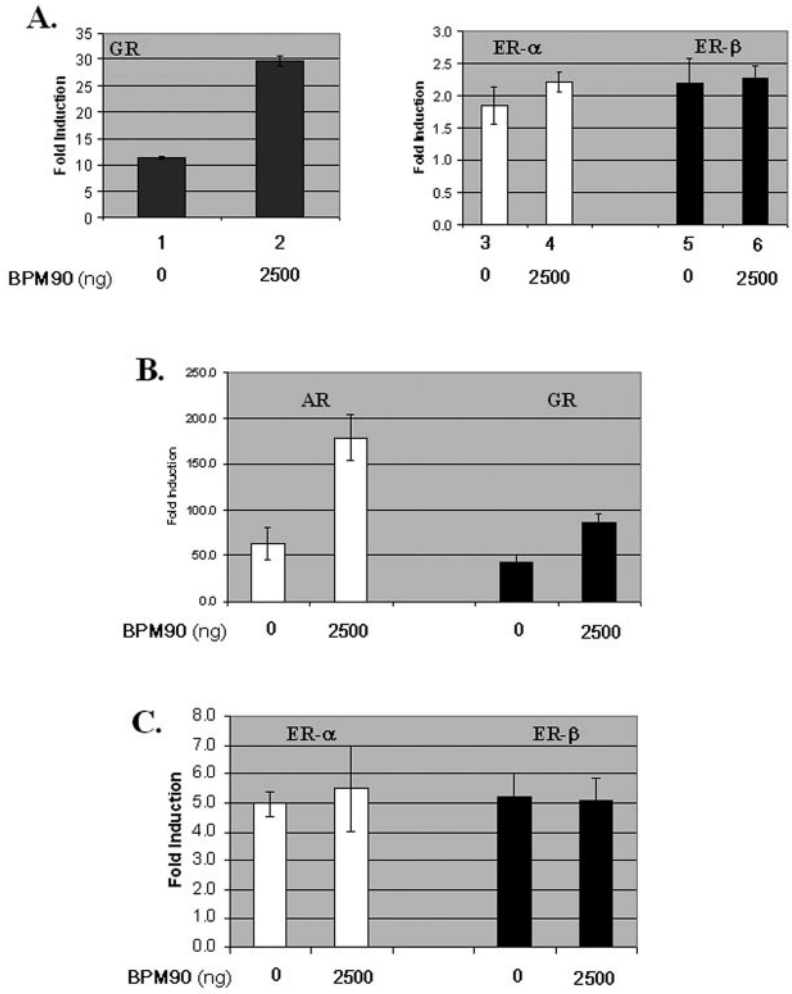

FIG. 6. **RanBPM enhances AR- and GR- but not ER-mediated transactivation.** *A*, PC3 cells were transfected with GR (*lanes 1* and *2*), ER-α (*lanes 3* and *4*), or ER-β (*lanes 5* and *6*) (250 ng/well), appropriate reporter plasmids (pARR3-tk-Luc for GR and pERE-Luc for ER-α and ER-β) (167 ng/well), and increasing amounts of pcDEBΔ-BPM90 (0, 250, or 2500 ng/well). Cells were induced in the presence or absence of 10 nM Dex or 10 nM E_2 for cells transfected with GR or ER, respectively, for 24 h prior to analysis for luciferase activity. Empty pcDEBΔ was used to keep DNA levels constant at 3 μg/well. Transfection efficiency was normalized with pRL-TK. *B*, LNCaP cells were transfected with pCMV/AR_6 (*lanes 1* and *2*) or pGR (*lanes 3* and *4*) (250 ng/well) and pARR3-tk-Luc as described previously. Receptor activity was determined in the absence (−) or presence (+) of pcDEBΔ-BPM90 (2500 ng/well). Transfected cells were induced in the presence of 1 nM R881 for AR, 10 nM Dex for GR, or vehicle alone for 24 h. Empty vector was used to ensure that DNA was kept constant at 3 μg/well. *C*, MCF7 cells were transfected with the ER-α or ER-β expression vector and pERE-Luc as described above and in the absence (−) or presence (+) of pcDEBΔ-BPM90 (2500 ng/well). Cells were treated with 10 nM E_2 or vehicle alone for 24 h prior to analysis for luciferase activity. DNA content was maintained at 3 μg/well with empty pcDEBΔ plasmid. For all experiments, protein content was used to normalize luciferase results (relative luciferase units/μg protein). Results are expressed at fold induction in the presence of ligand and are an average of three replicates. Results are representative of three independent experiments.

linked to the Ras/ERK signaling pathway because of its interaction with the MET receptor protein tyrosine kinase (31).

The three RanBPM library clones that were isolated in the yeast-two hybrid assay contain amino acid sequences that are upstream of the BPM55 start codon but are within the coding region of BPM90, which suggests that BPM90 interacts with AR *in vivo*. This finding was confirmed in LNCaP cells by co-immunoprecipitation of BPM90 with endogenously expressed AR (Fig. 2*B*). A SPRY domain straddles the start codon of BPM55 such that part of the domain is upstream of the start codon. Hence, the complete SPRY domain is not present in BPM55 but is fully intact in BPM90. The SPRY domain, originally identified in the ryanodine receptor, is involved in protein-protein interactions (32). It is probable that the SPRY domain is the region of BPM90 that interacts with the NH_2 terminus of the AR. In fact, an NH_2-terminal truncation of the SPRY domain results in the loss of the RanBPM/AR interaction in yeast (data not shown). The interaction between RanBPM and AR is also lost when the SPRY domain is truncated at the COOH-terminal end (Fig. 2*C*).

RanBPM may also have a role in Ran-dependent nuclear transport. Ran/ARA24 is a small nuclear Ras-like GTPase that is ubiquitously expressed (33). Quantitative analysis shows that $\sim 10^7$ molecules of Ran/ARA24 are expressed in an individual cell (34). The Ran/ARA24 nuclear import/export pathway is well defined and uses a RanGTP/GDP gradient along with carrier proteins to shuttle proteins across the nuclear membrane (reviewed in Refs. 35 and 36). Interestingly, Ran/ARA24 was also found to interact with the NH_2 terminus of the AR, suggesting that AR, RanBPM, and Ran/ARA24 work together as part of a multi-protein complex (34).

The LNCaP co-immunoprecipitation data presented in this paper show that AR interacts with BPM90 *in vivo* only in the presence of ligand when the receptor has been activated (Fig. 2*B*). Therefore, it is probable that RanBPM and Ran/ARA24 enhance AR activity either by promoting nuclear import of the activated receptor or by discouraging AR export from the nucleus. These mechanisms are currently under investigation.

The AR_{1-232} fragment that was used as bait in the yeast two-hybrid assay contains unique elements such as the long polymorphic polyglutamine (poly-Gln) and polyglycine (poly-Gly) repeats (37). The poly-Gln tract is of particular interest because an inverse relation has been demonstrated between the length of the poly-Gln tract and AR activity (38), which suggests that poly-Gln tracts have an important role in directing AR activity.

Our GST- and His-tagged pull-down assays have demonstrated that RanBPM interacts directly with AR_{1-232} and with larger AR fragments that span the full NH_2-terminal domain (AR_{1-559}) (Fig. 2, *A* and *C*). In addition, RanBPM was found to interact with the DBD of the AR, whereas the LBD did not contribute to the interaction (Fig. 2*C*). The overexpression of BPM90 in the PC3 cell line, which does not express endogenous AR, along with full-length AR resulted in a 3-fold increase in androgen-induced AR activity from a synthetic promoter, pARR3-tk-Luc, above basal AR activity (Fig. 4*A*). The functional domain of BPM90 is not located at the NH_2 terminus because BPM_L, which is truncated for the first 147 amino acids of BPM90, also enhances AR activity (Fig. 4*A*).

Transcription assays using the full-length AR indicate that

BPM90-mediated enhancement of AR activity is ligand-dependent because negligible activity was seen in the absence of hormone, even at very high levels of BPM90 (Fig. 4*B*). These results are consistent with the nuclear localization of BPM90 and suggest that AR must first be activated before RanBPM can function. The ability of BPM90 to augment AR-mediated transactivation is not only seen with the artificial ARR3 promoter, which has three ARR motifs in tandem, but also with the naturally occurring PSA promoter and, to a lesser degree, the rat probasin promoter (Fig. 5).

Furthermore, it seems probable that the RanBPM is most likely to influence the transcriptional activity of AF-1, which is found in the NH_2-terminal domain, and not the COOH-terminal AF-2 function. Additional transcriptional assays in PC3 cells using the constitutively active AR, AR_{1-646}, confirm the role of BPM90 in AF-1 transactivation because enhanced AR activity was observed irrespective of hormone (Fig. 4*C*). The ligand-dependent interaction between AR and BPM90 is confirmed because the co-immunoprecipitation from LNCaP cells only occurs with R1881 treatment (Fig. 2*B*). These data are consistent with the role of RanBPM in AR transport across the nuclear membrane because full-length AR requires activation by its cognate ligand before translocation, whereas AR_{1-646} is targeted to the nucleus even in the absence of hormone.

Although RanBPM interacts with the unique NH_2-terminal domain of the AR, it also interacts with the DBD (Fig. 2*A*). This finding suggests that RanBPM can interact with other steroid receptors as well. Transcription assays carried out in PC3 cells show that overexpression of BPM90 enhances the transactivation function of GR, whereas the activity of ER-α and ER-β remains unchanged (Fig. 6*A*). These results demonstrate that RanBPM selectively amplifies steroid receptor activity. These results are supported by observations with receptor transactivation assays using LNCaP and MCF7 cell lines (Fig. 6, *B* and *C*). Again, the role of RanBPM in receptor transport is supported by these data since GR, but neither ERα nor ERβ requires translocation to the nucleus upon activation. Because ER-α and ER-β are already localized in the nucleus (39, 40), the overexpression of RanBPM is not likely to have an effect on ER activity.

The data presented here show that BPM90 is capable of enhancing AR transactivation in a ligand-dependent manner. Nevertheless, the constitutively active form of AR is also influenced by the overexpression of BPM90. Furthermore, BPM90 influences the activity of GR but has no effect on either ER-α or ER-β activity, which suggests that this protein selectively directs steroid receptor activity, possibly at the level of nuclear transport. Nevertheless, the elucidation of RanBPM function will provide further insight into the mechanism of AR-mediated gene expression.

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RanBPM, a Nuclear Protein That Interacts with and Regulates Transcriptional Activity of Androgen Receptor and Glucocorticoid Receptor

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