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Jun Dimerization Protein 2 Functions as a Progesterone Receptor N-Terminal Domain Coactivator

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The progesterone receptor (PR) contains two transcription activation function (AF) domains, constitutive AF-1 in the N terminus and AF-2 in the C terminus. AF-2 activity is mediated by a hormone-dependent interaction with a family of steroid receptor coactivators (SRCs). SRC-1 can also stimulate AF-1 activity through a secondary domain that interacts simultaneously with the primary AF-2 interaction site. Other protein interactions and mechanisms that mediate AF-1 activity are not well defined. By interaction cloning, we identified an AP-1 family member, Jun dimerization protein 2 (JDP-2), as a novel PR-interacting protein. JDP-2 was first defined as a c-Jun interacting protein that functions as an AP-1 repressor. PR and JDP-2 interact directly in vitro through the DNA binding domain (DBD) of PR and the basic leucine zipper (bZIP) region of JDP-2. The two proteins also physically associate in mammalian cells, as detected by coimmunoprecipitation, and are recruited in vivo to a progesterone-inducible target gene promoter, as detected by a chromatin immunoprecipitation (ChIP) assay. In cell transfection assays, JDP-2 substantially increased hormone-dependent PR-mediated transactivation and worked primarily by stimulating AF-1 activity. JDP-2 is a substantially stronger coactivator of AF-1 than SRC-1 and stimulates AF-1 independent of SRC-1 pathways. The PR DBD is necessary but not sufficient for JDP-2 stimulation of PR activity; the DBD and AF-1 are required together. JDP-2 lacks an intrinsic activation domain and makes direct protein interactions with other coactivators, including CBP and p300 CBP-associated factor (pCAF), but not with SRCs. These results indicate that JDP-2 stimulates AF-1 activity by the novel mechanism of docking to the DBD and recruiting or stabilizing N-terminal PR interactions with other general coactivators. JDP-2 has preferential activity on PR among the nuclear receptors tested and is expressed in progesterone target cells and tissues, suggesting that it has a physiological role in PR function.

Progesterone receptor (PR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that play essential roles in tissue development, reproduction, and homeostasis. In the absence of ligand, PR is inactive and retained in oligomeric complexes with heat shock proteins (HSPs). Binding of ligand induces a conformational change in the receptor, resulting in dissociation from HSPs, dimerization, and interaction with specific progesterone response elements (PREs) present in the promoter region of target genes (47). DNA-bound PR is thought to activate transcription through recruitment of coactivator proteins, such as the family of steroid receptor coactivators (SRCs), CBP, or p300 CBP-associated factor (pCAF), and by direct or indirect protein interactions with the general transcription machinery (17, 23, 36).

Nuclear receptors share a modular domain structure consisting of a highly conserved DNA binding domain (DBD) and a highly conserved ligand binding domain (LBD), located centrally and at the C terminus, respectively, plus an N-terminal domain. The N-terminal domain is the most divergent region among nuclear receptors in both length and sequence and is involved in mediating receptor-specific functions (38, 56). Steroid hormone members of the nuclear receptor family have at least two autonomous transcriptional activation domains, a conserved, hormone-dependent activation function (AF) domain, AF-2, in the C-terminal LBD and a ligand-independent domain, AF-1, in the N-terminal region (55, 56). Interactions mediated by AF-2 in the LBD have been well characterized. This region forms a hydrophobic pocket in response to a ligand-induced conformational change, enabling receptor interaction with LXXLL amphipathic helix motifs present in SRCs (17, 19, 24, 36, 45). Interaction of SRCs with AF-2 results in recruitment of other SRC-associated coactivators, including CBP and pCAF, which possess histone acetyltransferase (HAT) activity, as well as the methyltransferase CARM1 (13). Clustering of AF-2-dependent chromatin remodeling HAT and methyltransferase activity at specific steroid hormone responsive promoters is thought to facilitate access for basal transcription factors. The sequence of the AF-1 domain is not conserved between receptors, and the protein interactions mediated by AF-1 are not well defined (28, 40, 42, 55).

PR is expressed in two isoforms transcribed from a single gene, full-length PR-B and N-terminally truncated PR-A (32). Aside from the N-terminal-most 164 amino acids (aa) of PR-B that PR-A lacks, these isoforms are identical in sequence and have similar ligand binding and DNA binding activities. However, PR-A is generally a weaker transcription activator than PR-B (21, 22, 25, 63). Under conditions in which PR-A lacks

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transactivation function, it can also act as a ligand-dependent repressor of PR-B as well as of other steroid receptors (39, 58). This repressive function of PR-A is dependent on an inhibitory domain (ID) located at the PR-A N terminus that is present in both receptors but is functional only in the A isoform (22, 25). PR-B contains an additional AF domain, AF-3, in the unique B-specific N-terminal region. AF-3 requires the PR DBD for transactivation and has minimal transcriptional activity when fused to a heterologous DBD. AF-3 is thought to contribute to the different transcription activities of PR-A and PR-B activities by inactivating the ID domain (49).

AF-1 is present in both A and B receptors and has been mapped to a minimal 91-aa sequence located immediately N terminal to the PR DBD (42). AF-1 is not rich in acidic amino acids and contains no recognizable secondary structure motifs. Activation of AF-1 is ligand independent, and it can function independently of AF-2, as demonstrated through mutations that disrupt AF-2 (42). However, within the context of full-length PR, AF-1 and AF-2 can synergize to elicit maximal hormone-dependent activity. PR N-terminal and LBD domains enhance each other's transcriptional activation in *trans* when expressed as separate polypeptides, and these domains interact directly in vitro as well (44, 53, 54). SRC and associated components of the coactivation complex (CBP and pCAF) can interact with the N terminus of PR and mediate functional enhancement (1, 44, 61). However, physical and functional interaction of SRC and associated components with N-terminal regions is much weaker than with AF-2, suggesting that AF-1 activity is mediated primarily by as yet undescribed protein interactions.

AF-1 is also thought to mediate partial agonist activity of the PR antagonist RU486. Although RU486 binding promotes PR dissociation from HSPs, dimerization, and interaction with DNA (2, 18, 20), it induces a distinct conformational change in the C-terminal tail of the LBD that inactivates AF-2, thereby preventing interaction with SRCs (2, 45, 57). Additionally, RU486 promotes an efficient interaction of PR with corepressors such as NCoR and SMRT that does not occur in the absence of ligand or in the presence of hormone agonist (29, 59). In certain contexts, however, RU486 exhibits a promoter-specific partial agonist activity that is specific to the B isoform and requires an intact AF-1 (41).

Because of the differences in activity between the A and B isoforms of PR as well as the lack of understanding of N-terminal domain protein interactions, we sought to isolate novel PR-interacting proteins responsible for activities mediated by the N-terminal domain. Using a *Saccharomyces cerevisiae* two-hybrid screening strategy, we identified a member of the AP-1 family, Jun dimerization protein 2 (JDP-2), as a novel PR-interacting protein that potentiates ligand-dependent PR transactivation predominantly by enhancing the N-terminal AF-1 activity. We present evidence to support a model in which JDP-2, through docking to the DBD, provides an alternate pathway independent of SRC-1 and AF-2 to recruit coactivators that work on the N-terminal AF-1 domain. JDP-2 preferentially coactivates PR among nuclear receptors tested and is expressed in female reproductive tissues and progesterone responsive cells, suggesting that it has a role in PR-specific functions.

MATERIALS AND METHODS

Materials. R5020 (Promegestone) was obtained from Dupont/New England Nuclear Products (Boston, Mass.). RU486 (Mifepristone), other steroid hormones, and thyroid hormone were purchased from Sigma (St. Louis, Mo.). Rabbit polyclonal antisera recognizing rat JDP-1 and JDP-2 have been previously described (3). An additional antiserum (962) was raised in rabbits immunized with purified recombinant rat JDP-2 expressed from a baculovirus vector in Sf9 insect cells. 1294/H9 is a mouse monoclonal antibody (MAb) that recognizes both A and B isoforms of human PR (15). MAb to six-histidine (1162/F6) or glutathione transferase (GST) (794/H2) tags have been previously described (8). Anti-FLAG M2 MAb was obtained from Sigma.

Bacterial expression plasmids. Bacterial vectors for expression of rat JDP-1 and JDP-2 as GST fusion proteins were constructed by PCR amplification of full-length cDNAs with primers designed to contain 5′ and 3′ unique restriction sites for insertion into *Bam*HI and *Eco*RI sites of pGEX2T (Amersham Pharmacia, Uppsala, Sweden). GST fusion proteins were expressed for 3 h at 37°C in *Escherichia coli* (BL21 cells; Stratagene, La Jolla, Calif.) after induction with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG; GibcoBRL, Gaithersburg, Md.). Cells were lysed by two freeze-thaw cycles in lysis buffer (50 mM Tris [pH 8.0], 250 mM KCl, 1% Triton X-100, 5 mM dithiothreitol [DTT], 5 μM $ZnCl_2$) and whole cell extracts (WCEs) prepared by centrifugation for 30 min at 100,000 × *g*.

Mammalian cell expression plasmids. Vectors for human and rat JDP-1 (rJDP-1) and rJDP-2, under the control of the cytomegalovirus promoter, were constructed by insertion of cDNAs into *Bam*HI and *Eco*RI sites of pCR3.1 (Invitrogen, Carlsbad, Calif.). pCDNA3-his-rat rJDP-2, containing polyhistidine-tagged JDP-2, was previously described (3). Domain constructs of JDP-2, including JDP-2/bZIP (aa 70 to 153), JDP-2ΔN (aa 70 to 195), and JDP-2ΔC (aa 1 to 153), were constructed by PCR amplification of rJDP-2 sequences and insertion into pCI-neo (Promega, Madison, Wis.). phPR-B expressing human PR-B under the control of the simian virus 40 (SV40) promoter has been previously described (58). PR domains under the control of the Rous sarcoma virus (RSV) promoter were cloned into pABΔGal (44), including PR-Bn-DBD (aa 1 to 639), PR-An-DBD (aa 165 to 639), and DBD-hinge plus LBD (hLBD) (aa 546 to 933). AF-1–DBD was constructed by PCR amplification of PR (aa 456 to 651) and subsequent insertion into pABΔGal. FLAG-PR-DBD-VP16 (PR aa 556 to 642) cloned into pCDNA1 was a gift from D. Shapiro (University of Illinois). Chimeric PR domain constructs in which the PR DBD was replaced by the Gal4 DBD were constructed by PCR amplification of PR sequences with primers containing *Bgl*II 5′ and *Bam*HI 3′ restriction sites, followed by insertion into pABΔGal in frame with PCR-amplified Gal4 DBD (aa 1 to 147). The resulting constructs included AF-1 (PR aa 456 to 562)–Gal4-DBD and Bn (PR aa 1 to 562)-Gal4-DBD. pABGalDBD-hLBD (PR aa 688 to 933) was a gift from S. Oñate (University of Pittsburgh). Vectors for other nuclear receptors included rat glucocorticoid receptor (GR) (RSV-GR; S. Nordeen, University of Colorado Health Sciences Center), human estrogen receptor (ER) (SVMT-ER; Carolyn Smith, Baylor), human thyroid hormone receptor (hTR) (hTRβ; M.-J. Tsai, Baylor) and human vitamin D receptor (VDR) (RSV-VDR; E. Allegretto, DuPont). Other cell expression plasmids were obtained as kind gifts, including pSG5-GRIP1 (M. Lazar, University of Pennsylvania), pCDNA3-c-Jun (A. Gutierrez-Hartmann, University of Colorado Health Sciences Center), and pCR3.1-SRC-1 (S. Oñate, University of Pittsburgh). Mammalian cell reporter gene constructs included PRE_2-TATA-Luc (Z. Nawaz, Baylor), mouse mammary tumor virus (MMTV)-pAH-Luc (S. Nordeen, University of Colorado Health Sciences Center), 5×-GalUAS-Luc (D. McDonnell, Duke University), DR-4 TRE (thyroid response element)-Luc (M.-J. Tsai, Baylor), vitamin D responsive element (VDRE) (1, 2), ΔMTV-Luc (E. Allegretto, DuPont), and ERE_3-TATA-Luc (D. McDonnell, Duke University).

Baculovirus expression vectors. Vectors with N-terminal polyhistidine tags for human PR-A and PR-B and various domains of PR, including DBD (PR aa 552 to 651), Bn-DBD (PR aa 1 to 688), An-DBD (PR aa 165 to 688), Bn (PR aa 1 to 534), An (PR aa 165 to 534), and hLBD (PR aa 634 to 933), were previously described (8, 53, 54). GST-PR-A, which was obtained from Bert O'Malley (Baylor), was also described previously (44). Vectors for GST-human JDP-1 (hJDP-1) and polyhistidine-tagged JDP-2 were constructed by in-frame insertion of cDNAs into G2T (Amersham Pharmacia) or pBluebachis2 (Invitrogen), respectively. GST-hJDP-1 was expressed in Sf9 cells and prepared as described below for bacterial expression of GST-JDP fusion proteins. All cloning junctures and deletion mutations were verified by DNA sequencing, and protein expression was confirmed by Western blotting. Proteins were expressed in Sf9 insect cells, and the cell lysis, extraction, and nickel resin affinity purification procedures used have been previously described (8, 9, 53, 54).

Cell culture and transfection. Cos-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) as previously described (8). T47D breast cancer cells, human endometrial carcinoma 1-B (HEC-1-B) cells, and HeLa cervical carcinoma and Chinese hamster ovary (CHO) cells were maintained in modified Eagle's medium supplemented with 5% FBS (HyClone) (18, 62). Cos-1 cells were plated in six-well dishes or 100-mm-diameter dishes at a density of 1.6×10^5 or 1.1×10^6 cells, respectively. HEC-1-B cells were plated at 2.25×10^5 cells/well in six-well dishes. At 24 h after plating, cells were transfected using Lipofectamine Plus reagents (GibcoBRL) according to the manufacturer's instructions. Following transfection, cells were grown in DMEM or DMEM supplemented with 5% charcoal-stripped FBS for 24 h and then treated in the same medium for 24 h with 10 nM (functional assays) or 100 nM (coimmunoprecipitation assays) R5020 or RU486.

In vitro GST pull-down protein interaction assays. Bacterial or Sf9 cell lysates containing free GST, GST–PR-A/GST–JDP-1 or GST–JDP-2 fusion proteins were incubated in suspension with 25 μl of glutathione Sepharose resins (Amersham Pharmacia) at 4°C for 1 h. After two washes in TEDG buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 1 mM DTT, 10% glycerol) plus 100 mM NaCl, resins with immobilized GST or GST-JDPs were incubated in suspension at 4°C for 1 h with baculovirus-expressed polyhistidine-tagged PR-A or PR-B or domains of PR. Resins were then washed and eluted with 2% sodium dodecyl sulfate (SDS)–β-mercaptoethanol sample buffer, and proteins in the eluates were detected by Western blotting. To map PR interaction sites within JDP-2, domain constructs of JDP-2 were expressed in Cos-1 cells by transient transfection. At 48 h after transfection, cells were lysed in EB buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 50 mM NaCl, 0.1% IGAPAL) and centrifuged at $13,000 \times g$ for 10 min to yield soluble WCE in the supernatant. WCEs were incubated with glutathione Sepharose beads containing immobilized free GST or GST–PR-A liganded with R5020. The incubation, washing, and elution conditions were as described above for pull-downs with GST–JDP-2, except that eluates were analyzed by Western blotting with JDP-2 antiserum (962). To detect GST–JDP-2 interaction with coactivators, [^{35}S]methionine-labeled proteins were synthesized in vitro by a coupled transcription-translation assay using the plasmid construct pCR3.1-c-Jun, pCR3.1-mCBP, pCR3.1-SRC-1, or SG5-GRIP1 (all containing a T7 promoter upstream of inserted cDNAs) and a TNT rabbit reticulocyte lysate expression system per the manufacturer's instructions (Promega). Labeled coactivators or baculovirus-expressed FLAG-pCAF was incubated with glutathione Sepharose-immobilized GST or GST–JDP-2, and the beads were washed and eluted as described above. Bound coactivators were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were soaked in 1 M sodium salicylate for 20 min prior to drying and exposure to autoradiography. Bound pCAF was detected by Western blotting with an anti-FLAG-tag antibody.

Coimmunoprecipitation assay. For coimmunoprecipitation of transfected PR and JDP-2, Cos-1 cells were plated at 1.1×10^6 cells per 100-mm-diameter dish. At 24 h after plating, cells were transfected with 1.5 μg of pCDNA1-PR-B and 0.75 μg of pCR3.1-JDP-2 expression plasmids by using Lipofectamine Plus and incubated for 72 h at 37°C. Prior to harvest, cells were incubated at 37°C for 2 h with vehicle (ethanol), R5020 (100 nM), or RU486 (100 nM). Cells were lysed in TEDG plus 0.4 M NaCl and centrifuged for 30 min at $100,000 \times g$ to prepare soluble WCE. Protein concentration was determined by the Bradford assay. WCE (1.0 mg) was diluted to 1 mg/ml in TEDG plus 100 mM NaCl and incubated for 4 h at 4°C in suspension with either JDP-2 or PR antibody or an unrelated control antibody immobilized to protein A- or protein G-Sepharose (Amersham Pharmacia). Resins were washed with TEDG plus 100 mM NaCl and eluted with SDS sample buffer, and the eluate was analyzed for PR by Western blotting with MAb 1294. For coimmunoprecipitation of endogenous PR and JDP-2, T47D cells were treated with R5020 and lysed as described above, except in TEDG without NaCl. Cell lysates were centrifuged to pellet nuclei and the nuclei were extracted for 1 h at 4°C with TEDG plus 0.4 M NaCl and 0.1% IGAPAL. A soluble nuclear extract was prepared as the supernatant of a $100,000 \times g$ centrifugation of the salt-extracted nuclei. The nuclear extract (NE) was diluted to 1.5 mg/ml in TEDG to give a final NaCl concentration of 100 mM, and the coimmunoprecipitation was performed as described above with 1.5 mg of NE.

ChIP. A6 is a subclone of T47D breast cancer cells that contains a stably integrated progestin-inducible MMTV-chloramphenicol acetyltransferase (CAT) reporter gene as previously described (43). Cells were plated in 150-mm-diameter culture dishes at a density of 8×10^6 cells/dish and grown for 5 to 7 days (80% confluence) in DMEM supplemented with 5% FBS. At 24 h prior to hormone treatment, cells were switched to DMEM plus 5% charcoal-treated FBS to deplete steroids and then treated in the same medium for 1 h at 37°C with a vehicle (ethanol) or 10 nM R5020. The chromatin immunoprecipitation (ChIP) assay was performed essentially as described by Lambert and Nordeen (33). Cell monolayers were fixed for 15 min at room temperature with 1% formaldehyde followed by quenching with 125 mM glycine and sonication in cell lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS) containing a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride [10 μg/ml], pepstatin [10 μg/ml], aprotinin [7.7 μg/ml]). Sonication was done at 4°C for eight rounds of 10 pulses each with, the sonicator (VWR Scientific) set for timer-hold duty cycle (for −90% output, −2). Lysates were centrifuged for 10 min at $100,000 \times g$ at 4°C and then precleared by incubation with a slurry of protein A-Sepharose. Antibody (15 μg) added to 1 ml of sheared cell lysate was incubated overnight at 4°C, and protein-DNA complexes were captured by adsorption to protein A-Sepharose (50 μl of packed beads). For JDP-2 rabbit antibody (962), protein A-Sepharose beads were used directly. For the PR MAb (1294), protein A Sepharose contained prebound rabbit anti-mouse (RAM) secondary bridging immunoglobulin B (IgB). The beads were washed in buffers containing 20 mM Tris-OH (pH 8.0), 1% SDS, 1% Triton X-100, 2 mM EDTA, and NaCl (either 150 or 500 mM). After final washes in 250 mM LiCl and TE buffer (10 mM Tris base [pH 8.0], 1 mM EDTA), protein-DNA complexes were eluted, the cross-links were reversed by heating to 65°C for 4 h, and the samples were digested with proteinase K (45°C for 1 h). DNA in the eluate was extracted, precipitated, and resuspended in 50 μl of distilled H_2O. Immunoprecipitated DNA (5 μl) and input-sheared DNA (5 μl) were submitted to 33 cycles of PCR with *Taq* polymerase and primer pairs that amplify a 224-bp region spanning the progesterone-glucocorticoid response elements (−214 to +10) of the proximal promoter of the C3H strain of MMTV (primer pair 5′-GCGGTTCCCAGGGCTTAAGT-3′ and 5′-GGACTGTTGCAAGTTTACTC-3′). Amplified PCR products were analyzed by ethidium bromide staining of 2% agarose electrophoresis gels. Agarose gels were scanned by a Fluor-S MultiImager (Bio-Rad), and the data were captured and quantitated using QuantityONE (Bio-Rad). Data were quantitated using ImageQuant 5.0 (Molecular Dynamics). Volume (pixels times intensity) of the PCR product of immune precipitated DNA was normalized to PCR of the corresponding input (ethanol or R5020). A number of controls were used to determine the specificity of the ChIP assay. Primers to a sequence (230 bp) in the MMTV-CAT plasmid, located 2,720 bp from the GRE/PREs of the promoter, served as a control for specificity of PR and JDP-2 association with the PRE/GRE promoter region. Both JDP-2 and PR were shown to have been immunoprecipitated from nuclear extracts of T47D cells by the 962 and 1294 antibodies, respectively, under ChIP assay conditions. The input PCR product amplified by the primer set specific for the MMTV GRE/PRE promoter was the same in the presence and absence of progestin treatment and, upon serial dilution, was in a linear range response range (data not shown).

SDS-PAGE and Western blotting. Proteins were separated by SDS–8 or 10% PAGE and analyzed by Western blotting as previously described (8, 15). Detection was by enhanced chemiluminescence (Amersham Pharmacia).

Cell-based nuclear hormone receptor-dependent transcription assays. Cells were plated at 1.6×10^5 (Cos-1) or 2.25×10^5 (HEC-1-B) per well in six-well culture dishes (Falcon, Franklin Lakes, N.J.) and were transfected 24 h later with constitutively active pCH110 β-galactosidase expression vector (as an internal control for transfection efficiency), PRE_2-TATA-Luc or MMTV-Luc progesterone-responsive luciferase reporter plasmids, and various amounts of PR expression vectors, together with pCR3.1-SRC-1, pCR3.1-JDP-1/pCR3.1-JDP-2, or JDP-2 domain constructs. pCR3.1 or pCI-neo empty vectors were added to maintain the moles of cytomegalovirus promoter constant. In experiments with PR constructs containing the LBD, cells were treated for 24 h prior to harvest with 10 nM R5020 or RU486 or with ethanol vehicle. In experiments with GR, ER, VDR, and TR, cells were transfected as described above with appropriate reporter target genes and treated with dexamethasone (100 nM), 17β estradiol (10 nM), 1,25-vitamin D_3 (10 nM) and thyroid hormone (T_3; 100 nM), respectively. At 48 h after transfection, cells were washed in buffer (40 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA) and lysed directly in the well using 0.3 ml of lysis buffer (20 mM K_2HPO_4 [pH 7.8], 5 mM $MgCl_2$, 0.5% Triton X-100). Lysates were centrifuged at $13,000 \times g$ for 5 min at 4°C to pellet cell particulates, and luciferase assays were performed as previously described, except using 10 μl of cell lysate (8, 20). Luciferase was normalized to β-galactosidase activity, and relative luciferase activity was calculated by setting the normalized values obtained with vehicle-treated reporter alone (i.e., no hormone and no PR) to 1.0 and all other values as severalfold increases relative to 1.0.

Northern blotting. Using a Purescript RNA isolation kit as recommended by the manufacturer (Gentra, Minneapolis, Minn.), total RNA was extracted from cells. Human female reproductive tract system I RNA blotting preparation (MassageBlot [for Northern blotting]) and human adult normal breast total RNA were obtained from Stratagene. For cell lines and breast RNA, equal amounts (25 μg) of total RNA were subjected to Northern blot analysis as previously described (10). Equal amounts of RNA transferred to the membrane

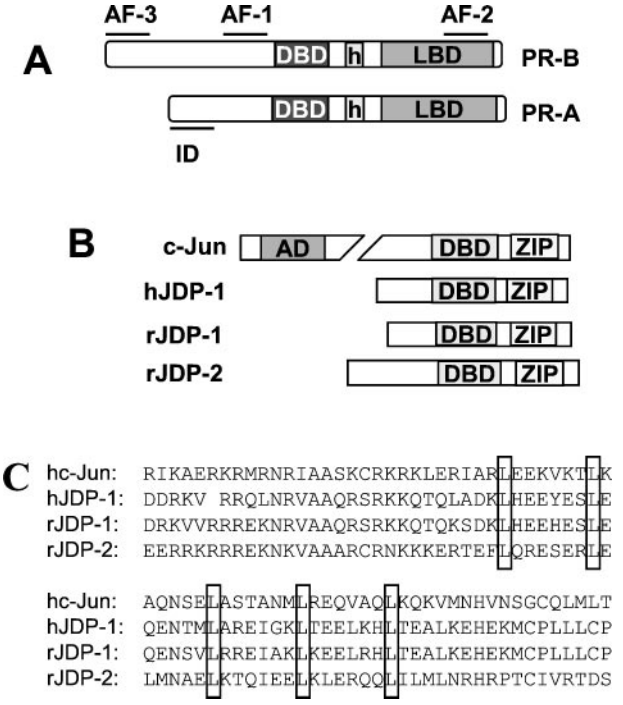

FIG. 1. (A) Domain structures of PR-A and PR-B isoforms. (B) Diagrammatic alignment with c-Jun of human and rat JDP-1 and rat JDP-2. (C) Sequence alignment of human c-Jun with human and rat JDP-1 and JDP-2 in the conserved basic and leucine zipper regions. Conserved leucines required for zipper-mediated dimerization are boxed.

were confirmed by methylase blue staining of 18S rRNA before hybridization. Northern blots were probed with rat JDP-2 cDNA labeled with ^{32}P by using the Rediprime-II random prime labeling system as described by the manufacturer (Amersham Pharmacia).

Electrophoretic gel mobility shift assay (EMSA). The DNA probe was a 32-bp double-stranded oligonucleotide containing a palindromic PRE derived from the MMTV long terminal repeat (18). Affinity-purified polyhistidine-tagged PR-A bound to R5020 was incubated in reaction buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 5 mM DTT, 2 mM $MgCl_2$, 10% glycerol, 32 μg of ovalbumin/ml [as a carrier protein]) for 1 h at 4°C with ^{32}P-labeled DNA (0.3 ng) in the presence or absence of 100 ng of affinity-purified JDP-2 in a total reaction volume of 25 μl. Reaction conditions included 1 μg of poly(dA-dT) as a nonspecific competitor DNA. Samples were electrophoresed on 5% native polyacrylamide gels as previously described (20). Gels were dried, and DNA-protein complexes were detected by autoradiography.

RESULTS

JDP-2 interacts directly with PR through the DBD of PR and the bZIP region of JDP-2. We used a previously described yeast two-hybrid strategy, with PR-A fused to the Gal4 DBD as bait, to screen a U2OS cell line human osteosarcoma library for novel gene products that interact in a hormone-dependent manner with the N-terminal half of PR (9). Positive PR-A interacting clones were further screened for interaction with the PR LBD fused to Gal4DBD (lacking N-terminal and DBD domains), and those that failed to interact were selected as candidate PR N-terminal-half interacting proteins (Fig. 1A). One interacting clone identified by this strategy contained a complete open reading frame (384 bp) for a 21,000 molecular weight gene product that has 70% amino acid identity to rat JDP-1. Rat JDP-1 and related JDP-2 are novel members of the AP-1 transcription factor family, originally isolated through interaction cloning with the leucine zipper of c-Jun (3). JDP-1 and JDP-2 are bZIP proteins that contain the leucine zipper common to AP-1 factors and the conserved basic amino acid region for DNA binding but lack the complete N-terminal activation domain (Fig. 1B and C). Both JDP-1 and JDP-2 bind DNA in vitro as Jun-JDP heterodimers and repress AP-1 transcription in cells. JDP-2 has also been shown to heterodimerize with and repress transactivation of other bZIP transcription factors including CCAAT/enhancer binding protein gamma (C/EBPγ) and ATF-2 (3, 31).

An in vitro GST pull-down assay was used to determine whether JDP-1 and JDP-2 interact directly with PR. These experiments were performed with hJDP-1 isolated from the yeast two-hybrid screen as well as with rat JDP-1 and JDP-2, as previously described (3). Recombinant baculovirus-expressed PR-A or PR-B isoforms, bound to the progestin R5020 and prepared as crude extracts from Sf9 cells, were incubated with GST fusions of human or rat JDP-1 and JDP-2 immobilized to glutathione Sepharose 4B resin. Figure 2A shows that PR-A and PR-B interacted efficiently with GST–JDP-1 (both human and rat) and GST–JDP-2 but not with the free GST control. Similar results were obtained with purified proteins, confirming that PR can interact directly with JDP-1or JDP-2 and does not require intermediary proteins (data not shown). Although PR-A/JDP-1 interaction in the yeast two-hybrid screening system in vivo was hormone dependent (data not shown), this interaction does not require ligand in vitro. PR-JDP interaction by GST pull-down assay in the presence of the progestin agonist R5020 was indistinguishable from that of unliganded PR or PR bound to antagonists RU486 and ZK98299 (data not shown). To map the region of PR required for interaction with JDP-1 and JDP-2, various baculovirus-expressed domains of PR were used in pull-down assays with GST–JDP-1 and GST–JDP-2. A construct containing the N-terminal region of PR-B (or of PR-A; data not shown) plus the DBD interacted with JDP-1 and JDP-2, as did the DBD alone. The PR-B (or PR-A; data not shown) N-terminal domain alone and C-terminal hLBD did not interact (Fig. 2B). These data localize JDP-1 and JDP-2 interaction to the DBD of PR.

Because JDP-2 stimulated PR activity in vivo (see Fig. 5) and JDP-1 did not (data not shown), additional experiments were performed with JDP-2 only. Domains of JDP-2 were used in GST pull-down assays to map the region of JDP required to interact with PR. The JDP-2 constructs were expressed by transient transfection in Cos-1 cells and analyzed in crude cell extracts for binding to immobilized GST–PR-A (occupied by hormone). Compared to full-length JDP-2, truncation of either the N or C domain of JDP-2 had little effect on association with PR (Fig 3A). Although a slight decrease in interaction was observed, the bZIP region alone is sufficient for interaction with PR (Fig. 3A). Further mapping within the bZIP region has not been done. These results indicate that protein-protein interaction between PR and JDP-2 is mediated through the DBD of PR and the bZIP region of JDP-2.

JDP-2 physically associates with PR and with the progesterone-responsive MMTV promoter in vivo. We initially examined the ability of exogenously expressed PR and JDP-2 to interact in cotransfected Cos-1 cells. Cos-1 cells lack PR expression and detectable expression of JDP-2 (see Fig. 6). Ex-

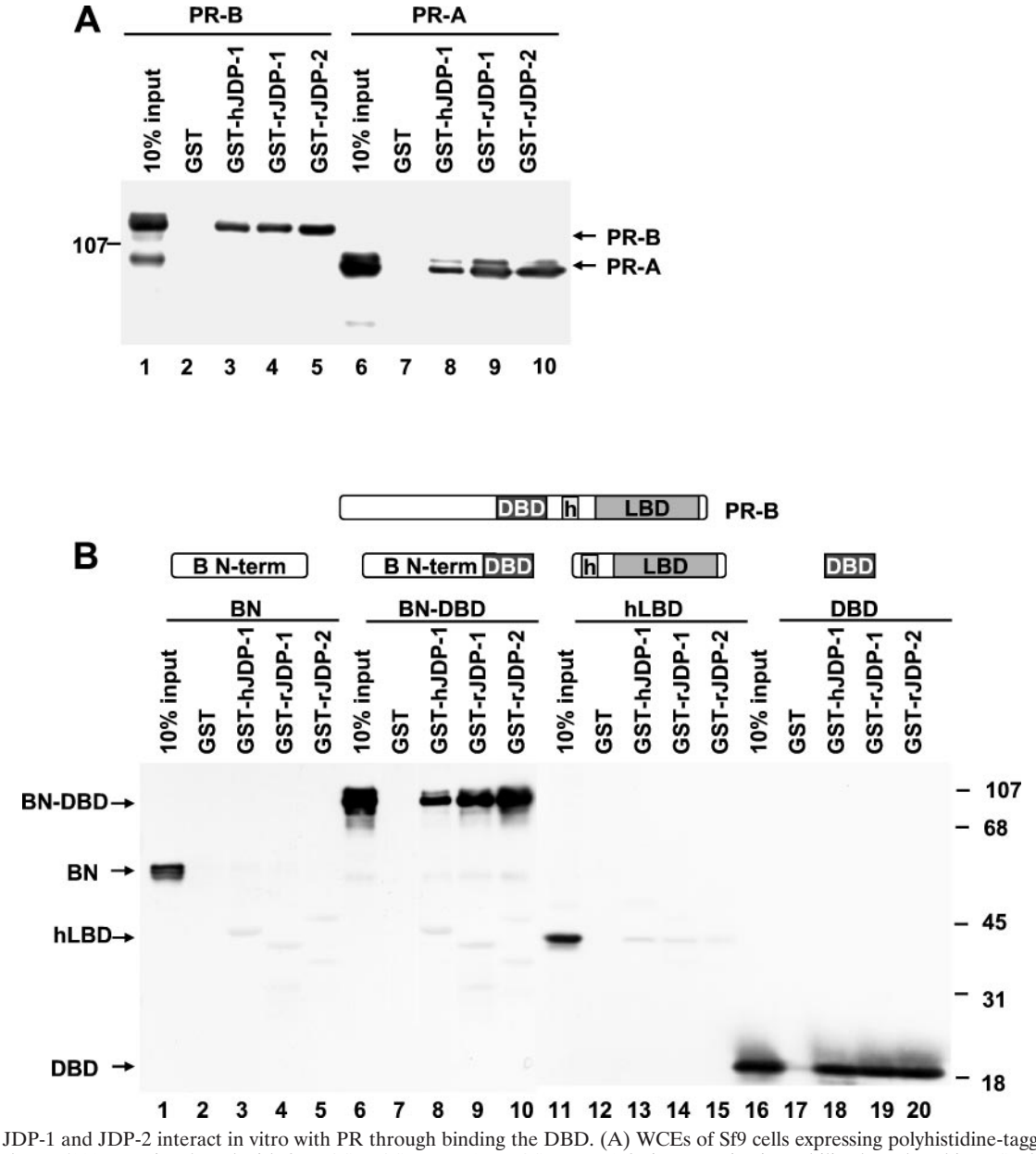

FIG. 2. JDP-1 and JDP-2 interact in vitro with PR through binding the DBD. (A) WCEs of Sf9 cells expressing polyhistidine-tagged PR-A or PR-B bound to R5020 were incubated with free GST, GST–JDP-1, or GST–JDP-2 fusion proteins immobilized to glutathione Sepharose resin. Resins were washed in a buffer containing 100 mM NaCl, and interacting proteins were eluted and analyzed by Western blotting with a MAb to the polyhistidine tag. Lanes 1 and 6 show the results for 10% input of PR-B and PR-A, respectively. Lanes 2 to 5 show the results for PR-B interaction with GST, GST–hJDP-1, GST–rJDP-1, and GST–rJDP-2 as indicated. Lanes 7 to 10 show the results for PR-A interaction with the same GST constructs in the same order as described for lanes 2 to 5. (B) Sf9 WCEs expressing polyhistidine-tagged domains of PR were incubated with immobilized GST or GST–JDPs as described above, and eluted proteins were detected by Western blotting. PR domain constructs are schematically represented above appropriate lanes. BN, input of PR-B N terminus (1); BN-DBD, input of B N terminus linked to the DBD (6). Inputs of hLBD (11) and DBD (16) are labeled. Lanes 2 to 5, 7 to 10, 12 to 15, and 17 to 20 represent results for interaction of the B N terminus, B N terminus plus DBD, hLBD, and DBD, respectively, with GST or GST–JDP constructs. Results are representative of at least three independent experiments.

tracts prepared from cells cotransfected with PR and JDP-2 expression vectors were incubated with antibodies specific to JDP-2 or PR, and immunoprecipitated proteins were detected by Western blotting. Coimmunoprecipitation of PR and JDP-2 was observed with either specific antibody, indicating a physical association between PR and JDP-2 in cells (Fig. 4A). Similar coimmunoprecipitation results were obtained from cells treated with vehicle (ethanol), R5020, or the antagonist RU486 (Fig. 4B). Thus, similar to in vitro pull-down assay results, PR–JDP-2 interaction as detected by coimmunoprecipitation from cells was ligand independent. We analyzed T47D breast cancer cells to determine whether endogenous JDP-2 and PR are capable of interacting in vivo. T47D are PR-positive cells (i.e., expressing both the A and B forms of PR) that also express JDP-2 (see Fig. 6B). Both the A and B forms of PR were specifically coimmunoprecipitated with the

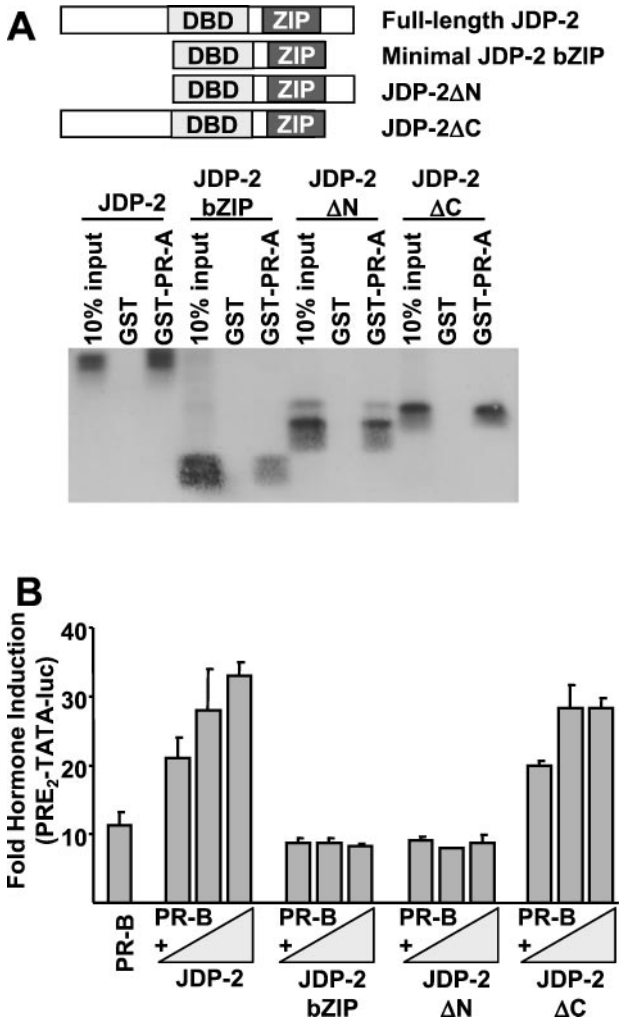

FIG. 3. PR interacts with the bZIP region of JDP-2. (A) Cos-1 cells were transfected with plasmids expressing JDP-2 or JDP-2 domain constructs, including bZIP, JDP-2ΔN, and JDP-2ΔC (2 μg of each plasmid). Lysates were analyzed by Western blotting with JDP-2 antibody to normalize pull-down inputs for equal expression of JDP-2 constructs. Lysates were incubated with GST or R5020-bound GST–PR-A immobilized to glutathione-Sepharose. Resins were washed as described in the Fig. 2 legend, and interacting proteins were eluted and detected by Western blotting with an antibody to JDP-2. (B) Cos-1 cells were transfected with phPR-B (0.35 ng) together with PRE_2-TATA-luc (200 ng) in the presence or absence of pCDNA.1-JDP-2 or JDP-2 domains in pCI-neo (25 to 200 ng). Cells were treated with vehicle or 10 nM R5020 for 24 h prior to harvest. Severalfold hormone induction was calculated as the ratio of relative luciferase activity of hormone-treated samples divided by the relative luciferase activity of corresponding vehicle-treated samples. Values are averages ± standard errors of the means (SEM) of three experiments.

JDP-2 specific antibody, and this also was not influenced by the presence of hormone (Fig. 4C).

A ChIP assay was used to determine whether endogenous JDP-2 and PR are recruited by promoters of progesterone regulated genes in vivo. For these experiments, a derivative of T47D cells (clone A6) containing a stably integrated MMTV-CAT gene was used (43). The MMTV promoter is highly inducible by glucocorticoids and progestins and contains multiple GRE/PREs, located between −190 and −80 bp from the transcription start site (Fig. 4D). A6 cells were grown for 2 days in steroid-depleted medium and were then treated for 1 h with vehicle (ethanol) or R5020 (10 nM). Cross-linked DNA fragments produced by sonication were immunoprecipitated with JDP-2 (962)- or PR (1294)-specific antibodies, and the immunoprecipitates were analyzed by PCR using pairs of specific primers spanning the PRE/GRE regions of MMTV (Fig. 4D). An unrelated RAM immunoglobulin G or an antibody to estrogen receptor (data not shown) was used as a control for nonspecific ChIP signals. Minimal occupancy of the MMTV promoter by PR, i.e., above the nonspecific background (RAM) of the ChIP assay, was detected in the absence of hormone. However, R5020 treatment resulted in a substantial increase of PR recruitment by the MMTV promoter (Fig. 4D). Quantitation of multiple independent ChIP experiments by phosphorimage analysis revealed that R5020 induced a fivefold increase of PR occupancy of the MMTV promoter. JDP-2 also associated with the MMTV promoter; however, occupancy by JDP-2 in the absence of hormone was slightly higher than that of PR and the severalfold increase induced by hormone (twofold) was less. The reason for the higher occupancy by JDP-2 in the absence of hormone is not clear. Some JDP-2 recruitment could be independent of PR. However, no detectable binding of JDP-2 to the region of the MMTV promoter amplified by PCR was detected in vitro by EMSA (data not shown). When immunoprecipitates were analyzed by PCR with primers for a region of the MMTV-CAT gene ~2,700 bp away from the promoter, no association of either PR or JDP-2 was detected, indicating the specificity of PR and JDP-2 recruitment to the MMTV promoter. These results highlight the finding that endogenous JDP-2 and PR are recruited in a largely hormone-dependent manner to a progesterone responsive promoter in the context of chromatin in vivo.

JDP-2 preferentially potentiates hormone-dependent PR-mediated gene transactivation. We next asked whether JDP-1 and JDP-2 might influence PR-mediated gene transactivation in cell-based transfection assays. The well-characterized hormone-dependent coactivation of PR by SRC-1 was used as a positive control (44, 45). PR-negative Cos-1 cells cotransfected with PR-B and a progesterone-responsive luciferase reporter gene (PRE_2-TATA-luc) exhibited a 10-fold induction of reporter gene activation in response to the progestin agonist R5020. Cotransfection of JDP-2 increased hormone-dependent PR transactivation up to another sevenfold. This stimulation of PR activity was dependent on the amount of JDP-2, and the magnitude of stimulation was similar to that of SRC-1 under the same conditions (Fig. 5A). Despite the fact that JDP-2 binds to PR in a ligand-independent manner, the effect of JDP-2 on PR-mediated transcription was hormone dependent. JDP-2 did not affect promoter activity in the absence of PR (data not shown) or in the presence of PR without ligand (Fig. 5A). Despite the close structural similarity between JDP-1 and JDP-2, JDP-1 had no effect on transactivation properties of PR in cotransfection assays (data not shown). Enhancement of PR activity by JDP-2 was not confined to a single cell type or promoter. Potentiation of progestin-induced activation of an MMTV-luciferase in Cos-1 cells was observed (Fig. 5B). Because JDP-2 has little-to-no effect on PR-dependent transactivation in the absence of ligand (Figs. 5A and B), the data in subsequent transfection experiments were pre-

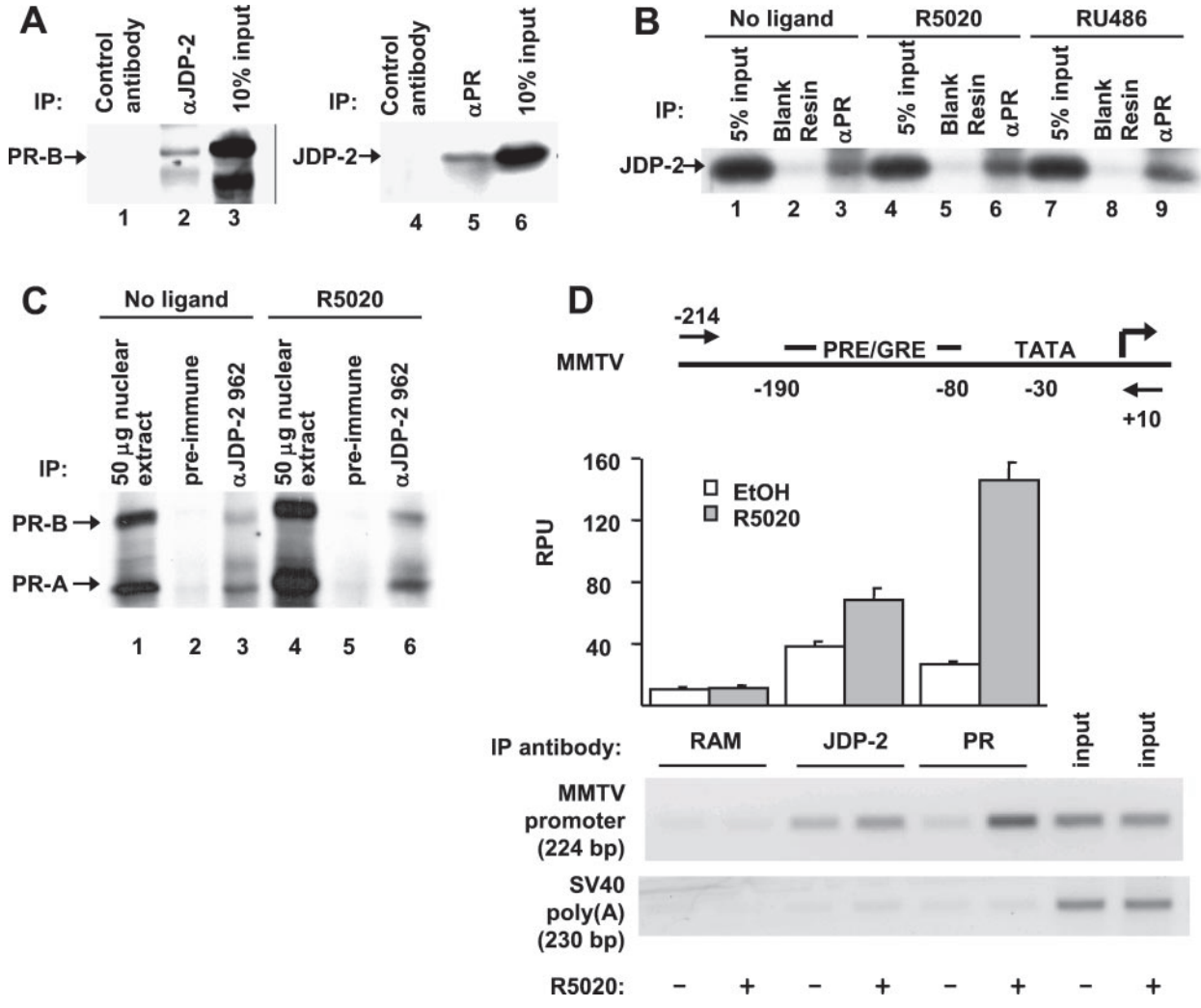

FIG. 4. JDP-2 and PR physically associate in mammalian cells and are recruited to a progesterone-inducible promoter in vivo. (A) Cos-1 cells were transfected with 1.0 μg of pCDNA1-PR-B and 1.5 μg of pCDNA3-hisJDP-2 and then treated with 100 nM R5020 for 1 h, followed by lysis in a high-salt buffer. Cleared WCEs were incubated with RAM control antibody or PR- or JDP-2-specific antibodies, using protein A-Sepharose as an adsorbent. Resins were washed in a buffer containing 100 mM NaCl, and bound proteins were detected by Western blotting with antibodies to PR or JDP-2. Lanes 1 and 4 show the results of PR and JDP-2 immunoprecipitation with a control unrelated antibody, respectively. Lane 2 shows the results of PR immunoprecipitation with JDP-2 antisera. Lane 5 shows the results of JDP-2 immunoprecipitation with MAb 1294 to PR. Lanes 3 and 6 show the results with 10% input of PR-B and JDP-2, respectively. (B) Cos-1 cells were transfected with 1.5 μg of pCDNA1-PR-B or 0.75 μg of pCR3.1-JDP-2, treated for 2 h with vehicle or 100 nM R5020 or RU486, and then lysed in high-salt buffer. Cleared WCEs were incubated with PR-specific MAb 1294 using protein G-Sepharose as an adsorbent or blank protein G-Sepharose resin as a control, and bound proteins were detected as described above. Lanes 1, 4, and 7 show the results of 10% input of PR-B in the presence of vehicle (ethanol), R5020, and RU486, respectively. Lanes 2, 5, and 8 show the results for nonspecific JDP-2 binding to blank resins alone. Lanes 3, 6, and 9 show the results for JDP-2 immunoprecipitation with the PR-specific MAb 1294 without ligand and in the presence R5020 and RU486, respectively. (C) T47D cells were hormone treated as described for panel A and lysed in buffer without salt. Nuclei were isolated by centrifugation and lysed in high-salt buffer. Cleared nuclear extracts were incubated with preimmune or JDP-2 specific antiserum, using protein A-Sepharose as an adsorbent. Resins were washed and eluted as described above, and bound PR was detected by Western blotting with an antibody to PR. (D) A6 cells were treated for 1 h with vehicle or 10 nM R5020, cross-linked with formaldehyde, and lysed in buffer containing SDS. Lysates were sonicated to shear chromatin and cleared by centrifugation. Clear lysates were incubated with a control unrelated antibody (FSG-RAM) or antibodies to PR or JDP-2, using protein A Sepharose as an adsorbent. Resins were washed in multiple buffers containing various salts and detergents, followed by elution, reversal of cross-links, and isolation of DNA fragments. Immunoprecipitated DNA fragments were analyzed by PCR using primers specific to the MMTV promoter of the integrated reporter gene or to the SV40 poly(A) 2,700-bp fragment removed from the promoter. Amplified products were normalized to similarly amplified input DNA of the corresponding treatment groups (vehicle or R5020). Relative phosphorimage units (RPU) were calculated as the ratio of volume (pixels × intensity) × 100 of immunoprecipitated DNA to the volume of corresponding input DNA, and values are averages ± SEM of four independent experiments.

sented as severalfold hormone induction rather than as relative luciferase activity. JDP-2 also increased PR transactivation of PRE $_2$-TATA-luc in HEC-1-B human uterine carcinoma cells (Fig. 5C) and in CHO cells (data not shown).

The effect of the presence of JDP-2 on gene transactivation properties of other nuclear receptors was also examined. As shown in Fig. 5D, JDP-2 had no influence on most of the other nuclear receptors tested, including ER, VDR, TR and andro-

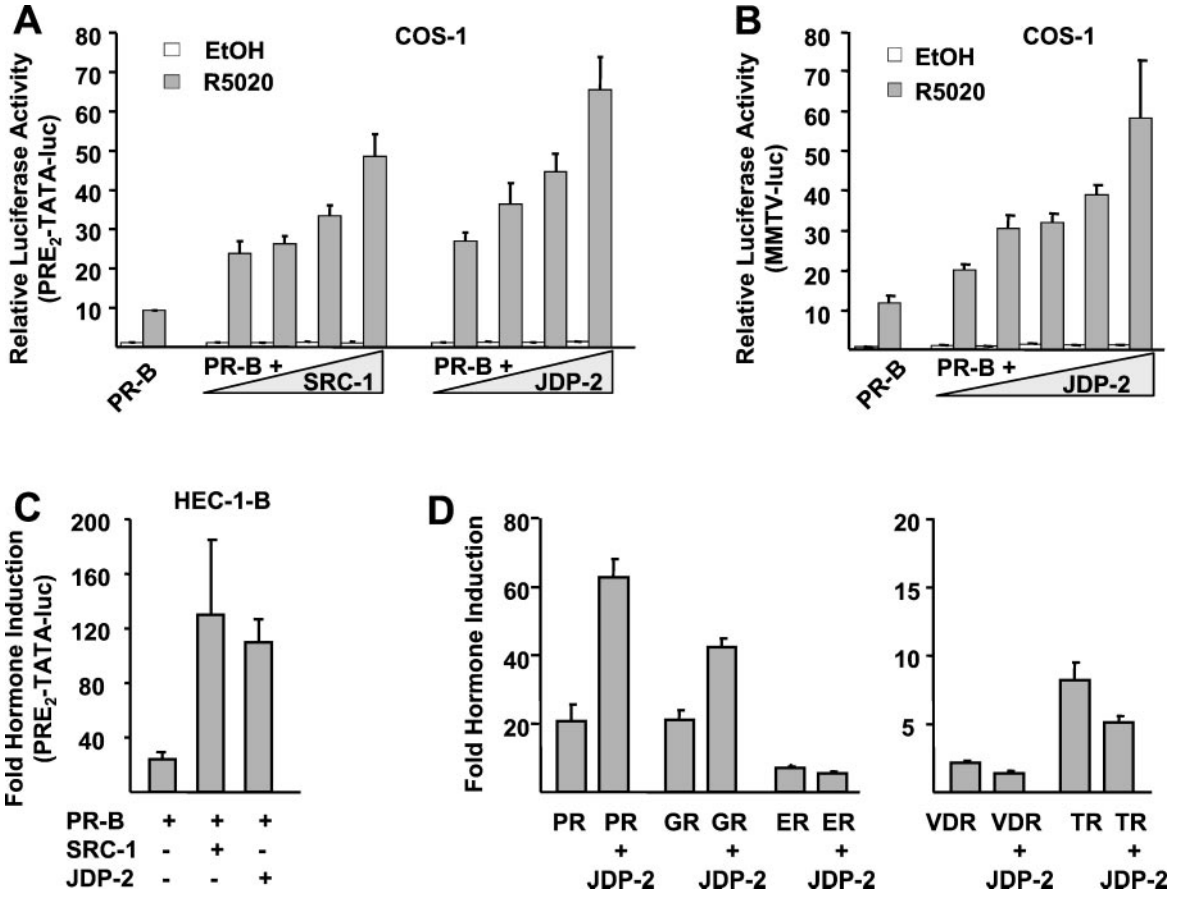

FIG. 5. JDP-2 increases ligand-dependent PR-mediated transactivation. (A) Cos-1 cells were transfected with phPR-B (1.5 ng) and the progesterone-responsive reporter PRE_2-TATA-Luc (200 ng) in the presence or absence of increasing amounts of pCR3.1-SRC-1 or pCDNA3-hisJDP-2 (25, 50, 100, and 200 ng). Cells were treated with the vehicle or 10 nM R5020 for 24 h prior to harvest. Relative luciferase activity was calculated by setting normalized luciferase activity of the reporter alone to 1.0 and all other treatment group values as severalfold relative to 1.0. Values are averages ± SEM of three independent experiments. (B) Cos-1 cells were cotransfected as described for panel A above with PR-B and JDP-2, except for the use of a different progesterone-responsive reporter gene, MMTV-Luc. (C) HEC-1-B cells were transfected with phPR-B (2.5 ng) and PRE_2-TATA-luc (200 ng) in the presence or absence of pCR3.1-SRC-1 (50 ng) or pCR3.1-JDP-2 (34 ng) and were hormone treated as described above. Severalfold hormone induction was calculated as the ratio of the relative luciferase activity of hormone-treated samples divided by the relative luciferase activity of corresponding vehicle-treated samples. Values are averages ± SEM of three independent experiments. (D) Cos-1 cells were transfected with indicated nuclear receptors [phPR-B (1 ng), pRSV-GR (2 ng), SVMT-ER (0.1 ng), TRβ (0.5 ng), or RSV-VDR (1.5 ng)], together with 200 ng of their cognate hormone-responsive luciferase reporter genes [PRE_2-TATA-luc (PR and GR), ERE_3-TATA-luc (ER), TRE-luc (TR), or VDRE(1,2)-ΔMTV-luc (VDR)] in the presence or absence of pCR3.1-JDP-2 (110 ng). At 24 h prior to harvest, cells were treated with the appropriate ligands: 10 nM R5020 (PR), 100 nM dexamethasone (GR), 10 nM estradiol (ER), 100 nM T_3 (TR), or 10 nM 1,25-vitamin D_3. Severalfold hormone induction was calculated as described for panel C, and values are averages ± SEM of three independent experiments.

gen receptor (AR) (S. Oñate et al., unpublished data). Under the same conditions, only the closely related GR was affected by JDP-2, and it showed a lower activity for GR than PR (Fig. 5D). Thus, among the nuclear receptor superfamily members examined, strong JDP-2 potentiation of transcription activity was preferential for PR.

JDP-2 is expressed in progesterone target tissues and cells. Tissue distribution of JDP-2 expression has not been well characterized. Available information showed that JDP-2 is expressed in a wide range of tissues and cell lines and that the relative level varies considerably (3, 31). Whether JDP-2 is expressed in progesterone target tissue has not been reported. Female reproductive tissues known to express PR were examined by Northern blotting for the presence of JDP-2 mRNA. A predominant single ~2.4-kb transcript was detected in most of the tissues examined, including human cervix, ovary, uterus, vulva, and breast (Fig. 6A). Placenta contained multiple JDP-2 transcripts of 7.0 kb, 2.4 kb, and 1.5 kb (Fig. 6A). Multiple JDP-2 mRNAs ranging from 1.0 to 7.0 kb were reported to be expressed in other nonendocrine target tissues, with the most abundant species being the 2.4- and 3.0-kb RNAs (31). The reason for the lack of multiple JDP-2 transcripts in our analysis is not known. JDP-2 protein and mRNA expression were also examined in various cell lines. As shown by Western blotting, JDP-2 protein is expressed highly in T47D breast cancer cells and HeLa cervical cancer cells, at a lower level in CHO cells, and minimally or not at all in Cos-1 cells (kidney) and HEC-1-B cells. Cos-1 cells transiently transfected with a JDP-2 expression vector were used as a positive control for Western blotting (Fig. 6B). The variation observed in relative levels of

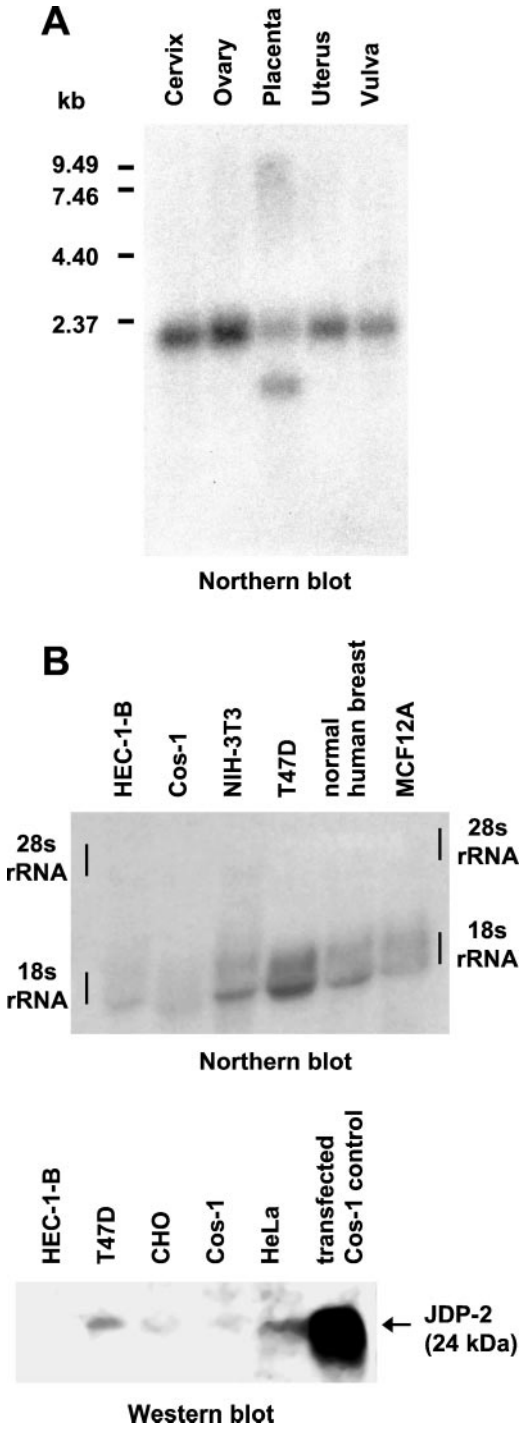

FIG. 6. JDP-2 is expressed in progesterone target tissues and variably expressed in cell lines. (A) JDP-2 mRNA was detected in human tissues by Northern blotting of a human female reproductive system (MessageBlot; Stratagene) using ^{32}P-labeled rat JDP-2 cDNA. (B) JDP-2 mRNA present in total RNA (25 μg) from indicated cell lines was analyzed by Northern blotting using ^{32}P-labeled JDP-2 cDNA. (C) Nuclear extracts (300 μg) of indicated cell lines were prepared as described for Fig. 4C and analyzed for JDP-2 expression by Western blotting with antibody to JDP-2. Extracts (10 μg) of Cos-1 cells transfected with pCR3.1-JDP-2 served as a positive control.

JDP-2 protein between cell lines correlated with the ability of cotransfected JDP-2 to potentiate PR-mediated gene transactivation. Exogenous JDP-2 had minimal activity on PR in cells with the highest levels of endogenous JDP-2 (T47D and HeLa), intermediate activity in CHO cells, and the highest activity in Cos-1 and HEC-1-B cells, which have the lowest levels of JDP-2 (data not shown). In selected cell lines, a correlation was observed between relative levels of JDP-2 mRNA and JDP-2 protein (Fig. 6B). By use of quantitative Western blotting, we compared cell extracts against a standard curve generated with purified PR and JDP-2. JDP-2 was estimated to be a low-abundance protein in T47D cells (0.001% of total protein) relative to PR level (0.025%), suggesting that JDP-2 is limiting with respect to PR. These data showing expression of JDP-2 in progesterone target tissues and cells suggest that it has a physiological role in PR action.

JDP-2 potentiates PR-mediated transcription predominantly through N-terminal AF-1. To determine which region of PR mediates enhancement by JDP-2, we compared the effects of JDP-2 in cotransfection assays on the activity of PR constructs containing either the hinge plus LBD (AF-2) or the N-terminal region of PR-B (AF-1 and AF-3) linked to the PR DBD. The AF-2 hLBD construct activated transcription approximately fivefold upon addition of R5020, while coexpression of JDP-2 increased hormone-dependent PR-mediated transactivation another 3.2-fold at the highest dose of JDP-2. SRC-1 gave a similar 2.6-fold stimulation (Fig. 7A). The PR-B N-terminal DBD construct constitutively activated the PRE_2-TATA-luc reporter gene by approximately 12-fold above basal promoter activity, while JDP-2 coexpression boosted this activity in a dose-dependent manner by another 14.7-fold at the highest concentration (Fig. 7A). SRC-1 gave a much lower 2.4-fold enhancement of transactivation mediated by the N-terminal domain (Fig. 7A). These results show that JDP-2 can independently enhance transactivation by either AF-2 or the N-terminal region of PR but more strongly affects the N terminus. While the magnitude of JDP-2 stimulation of AF-2 activity is similar to that of SRC-1, JDP-2 is much more active on the N terminus than SRC-1 (Fig. 7A).

Because the PR-B N-terminal construct contains both AF-1 and AF-3, we sought to determine which activation domain mediates the effect of JDP-2 by comparison of the PR-A and PR-B N-terminal regions linked to the DBD. Both N-terminal DBD constructs constitutively activated transcription to similar levels under the conditions of the assay, and the activity of each was strongly and equally stimulated by JDP-2 (Fig. 7B). Again, the strong enhancement by JDP-2 (14-fold) contrasted with the weak twofold effect of SRC-1 (data not shown). These results show that JDP-2 does not require AF-3 to potentiate transcription activity of the PR N-terminal domain, suggesting that it acts predominantly on AF-1. To determine whether AF-1 is the minimal region of the N-terminal domain required for enhancement by JDP-2, we next analyzed the influence of JDP-2 on a construct containing the PR-DBD linked to the previously defined (42) minimal AF-1 (PR aa 456 to 546). As shown in Fig. 7C, JDP-2 increased the constitutive activity of AF-1–DBD in a dose-dependent manner. However, the severalfold increase (4.4-fold) was lower than that observed with entire N-terminal DBD fragments (Fig. 7B and C) but still greater than the effect observed on the hLBD AF-2 (Fig. 7A).

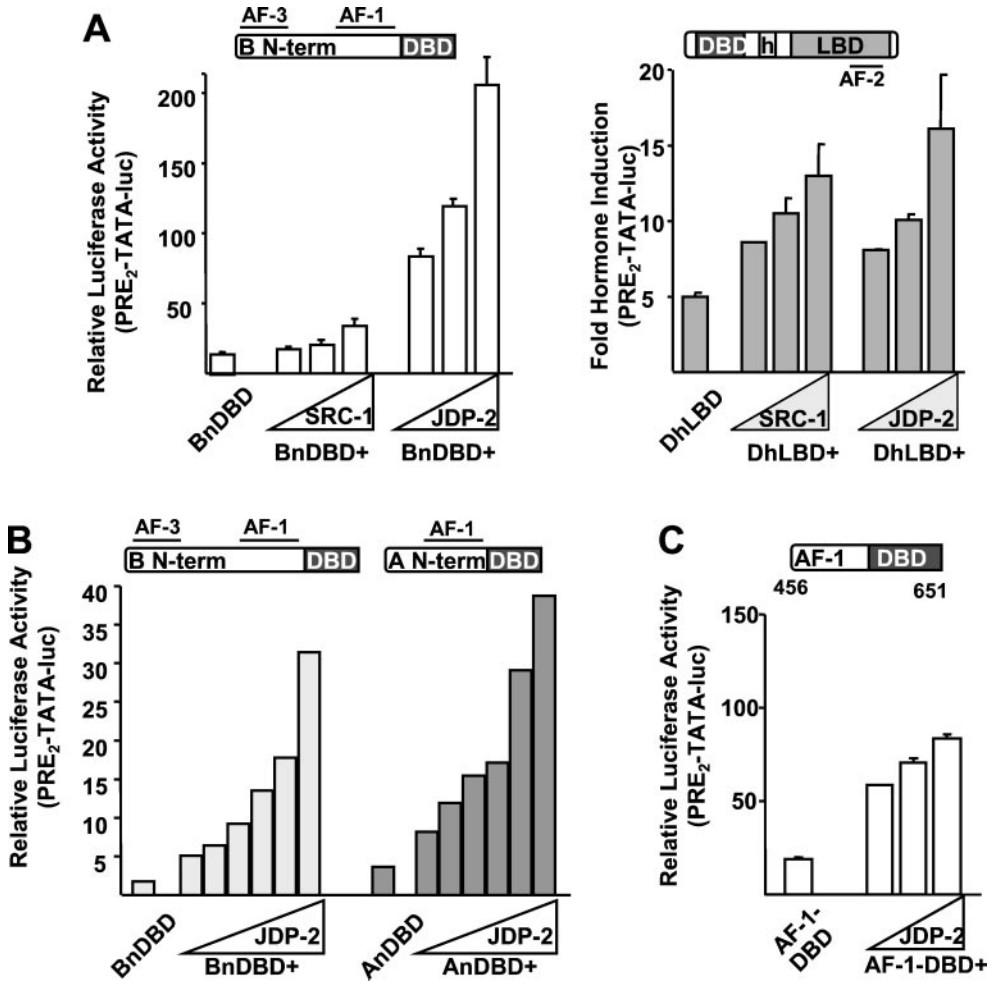

FIG. 7. JDP-2 enhances transactivation of the N-terminal AF-1 domain of PR. Cos-1 cells were transfected with the PR domain constructs indicated together with PRE_2-TATA-luc reporter (200 ng) in the presence or absence of various amounts of pCR3.1-SRC-1 or pCR3.1-JDP-2. Equimolar doses of SRC-1 and JDP-2 expression vectors were used (50 to 200 ng or 34 to 137 ng of SRC-1 or JDP-2, respectively). Cells transfected with DhLBD were treated with vehicle or 10 nM R5020 24 h prior to harvest. Other constructs lacking the LBD did not receive hormone. Relative luciferase activity and severalfold hormone induction were calculated as for Fig. 5. Cos-1 cells were transfected with BnDBD (10 ng) or DhLBD (50 ng) (A), with BnDBD (1.5 ng) or AnDBD (10 ng) (B), or with AF-1–DBD (20 ng) (C). Values are averages ± SEM of at least three independent experiments.

The N-terminal domain of JDP-2 is required for potentiation of PR-mediated gene transactivation. Because JDP-2 was initially reported to be a repressor of AP-1 (3), it was important to determine the region of JDP-2 required for its stimulatory effect on PR. Cos-1 cells were cotransfected with PR-B, the PRE_2-TATA-luc reporter gene, and the same domains of JDP-2 shown in Fig. 3 to map the region of JDP-2 that mediates protein interaction with PR. Although the bZIP region alone is sufficient for in vitro interaction with PR (Fig. 3A), this region has no activity on PR-mediated transcription in vivo (Fig. 3B). A C-terminal truncation mutant stimulated PR activity to a level similar to that stimulated by full-length JDP-2; however, an N-terminal truncated JDP-2 had no activity on PR (Fig. 3B). These results indicate that the bZIP region and the N-terminal domain of JDP-2 are required together to increase PR activity, whereas the C-terminal domain is dispensable.

The PR-DBD is necessary but not sufficient for functional response to JDP-2. In vitro binding data indicated that the DBD of PR mediates protein interaction with JDP-2 (Fig. 2). To determine whether the DBD is required for JDP-2 stimulation of PR-mediated gene transactivation in vivo, we examined the effect of the presence of JDP-2 on the activity of receptor chimeras containing the Gal4 DBD linked to either the entire N-terminal domain of PR-B, minimal AF-1, or the C-terminal hLBD that harbors AF-2. Transactivation was examined with a luciferase reporter gene containing multimerized Gal4 upstream response sequences. As shown in Fig. 8A, JDP-2 had minimal effect (twofold increase of reporter gene) on the PR-B N terminus linked to Gal4 DBD compared to that of the N-terminal domain (14-fold) linked to its own DBD (Fig. 7A). No effect of JDP-2 was observed on AF-1 or hLBD (AF-2) domains when linked to the Gal4 DBD (Fig. 8A). In contrast, SRC-1 stimulated activity of the Gal4 DBD-hLBD chimera to the same extent as the PR DBD-hLBD (data not shown). SRC-1 stimulation was expected, as the Gal4 DBD-hLBD chimera contains the AF-2 interaction site for SRC-1. Thus, the specific PR-DBD protein interaction site for JDP-2 appears to be required for JDP-2 to stimulate PR activity in

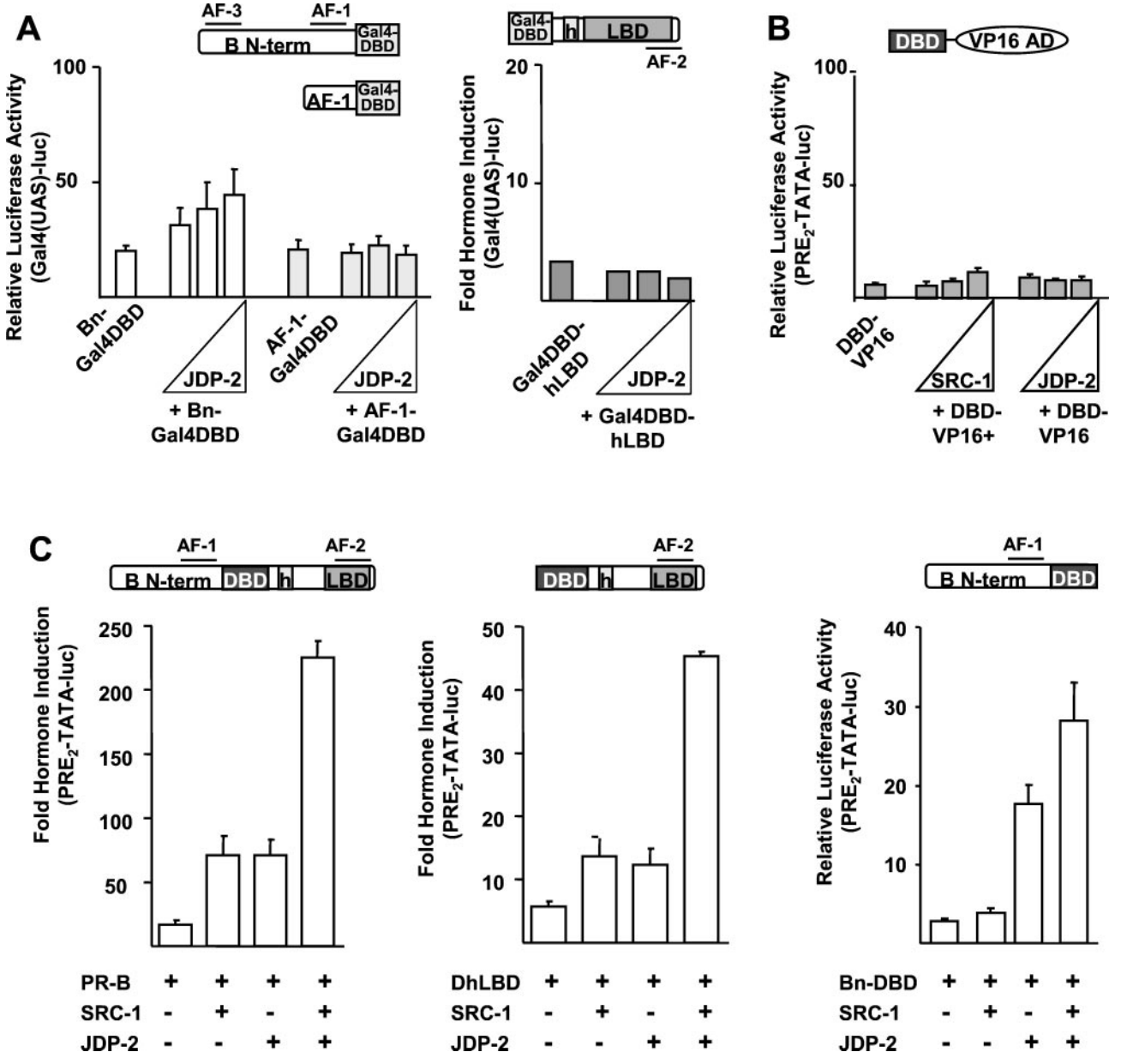

FIG. 8. PR-DBD is required, but not sufficient, for JDP-2 enhancement of PR transactivation domains, while JDP-2 and SRC-1 synergize to coactivate AF-2 but not the N terminus. (A) Cos-1 cells were cotransfected with the Gal4DBD-PR chimeric constructs indicated, including BnGal4DBD (1 ng), AF-1–Gal4DBD (1 ng), or GalDBD-LBD (50 ng), together with the 5×-GalUAS-luc reporter (500 ng), in the presence or absence of various amounts of pCR3.1-JDP-2 (34 to 137 ng). Cells transfected with GalDBD-LBD were treated with vehicle or 10 nM R5020 24 h prior to harvest. Constructs lacking the LBD did not receive hormone. (B) Cos-1 cells were transfected with pfPR-DBD-VP16 (50 ng) together with PRE_2-TATA-luc (200 ng) in the presence or absence of pCR3.1-SRC-1 (50 to 200 ng) or pCR3.1-JDP-2 (34 to 137 ng). (C) Cos-1 cells were transfected with phPR-B (1.5 ng) (leftmost panel), DhLBD (50 ng) (middle panel), or BnDBD (1.5 ng) (rightmost panel), together with PRE_2-TATA-luc (200 ng), in the presence or absence of pCR3.1-SRC-1 (200 ng) or pCR3.1-JDP-2 (137 ng). Cells transfected with PR-B or DhLBD were treated with vehicle or 10 nM R5020 for 24 h. Relative luciferase activity and severalfold hormone induction values were calculated as for Fig. 5. Values are averages ± SEM of at least three independent experiments.

vivo. The minimal region of PR stimulated by JDP-2 was a construct containing the DBD and adjacent AF-1 (Fig. 7). To determine whether AF-1, together with PR DBD, is required specifically or whether JDP-2 can affect activity through the DBD associated with a heterologous activation domain, we next examined the influence of JDP-2 on the constitutive activity of a chimera containing the DBD of PR linked to the VP16 activation domain (Fig. 8B). JDP-2 had no effect on the activity of the PR-DBD-VP16 construct and neither did SRC-1 (Fig. 8B). The SRC-1 result was expected, as the PR-DBD-VP16 construct lacks the protein interaction sites for SRC-1. These results indicate that the PR DBD and its own AFs are required for response to JDP-2 and that neither domain alone is sufficient. The fact that a heterologous activation domain

(VP16) was not able to substitute for AF-1 suggests that a specific interaction between the PR-DBD and AF-1 is essential for JDP-2 to exert an effect on PR.

JDP-2 synergizes with SRC-1 on AF-2 but functions independently of SRC-1 on AF-1. SRC-1 has been reported to physically interact with and stimulate the N-terminal AF-1 activity of PR and other steroid receptors (1, 37, 44, 61). However, SRC-1 interactions with AF-1 are generally weaker than with AF-2. In contrast, JDP-2 has much stronger activity for PR AF-1 than AF-2 (Fig. 7). To determine whether JDP-2 affects PR AF-1 activity in an SRC-1-dependent or -independent manner, we examined the influence of JDP-2 and SRC-1 together compared to that of each protein alone. Cotransfection of cells with JDP-2 and SRC-1 together increased hormone-dependent transactivation mediated by PR-B in a manner that was more than additive of the effects of either protein alone (Fig. 8C). A subthreshold level of SRC-1 and JDP-2 alone each increased PR-B transactivation approximately fourfold, whereas the same doses of SRC-1 and JDP-2 together gave a 14-fold activation (Fig. 8C, leftmost panel). When PR truncation constructs were analyzed, JDP-2 and SRC-1 also exhibited synergistic activity on the C terminus (AF-2) (Fig. 8C, center panel), but the two proteins together increased the activity of the N-terminal activation domains in an additive manner (Fig. 8C, rightmost panel). These results suggest that the strong potentiation of AF-1 activity by JDP-2 occurs independently of SRC-1 and that weaker effects on AF-2 involve SRC-1-mediated pathways.

JDP-2 coexpression potentiates the partial agonist activity of the PR antagonist RU486. The PR antagonist RU486 inactivates receptor by inducing an altered conformation in the C-terminal tail of the LBD that prevents binding of SRCs to AF-2 (2, 57, 60). However, RU486 is not a pure antagonist and can exhibit partial agonist activity, depending on the cellular and target promoter context (6, 41, 50). The partial agonist activity of RU486 is thought to be mediated through the N terminus and has previously been demonstrated to require an intact AF-1 (41). Because JDP-2 exhibits strong activity independently on the N-terminal domain AF-1, we asked what effect JDP-2 might have on PR-mediated transcription in response to the presence of RU486. In Cos-1 cells transfected with PR-B alone, no induction of PRE_2-TATA-luc was observed by treatment with RU486 (Fig. 9). Cotransfection of SRC-1 had no effect, whereas JDP-2 resulted in a fivefold RU486 induction of PRE_2-TATA-luc expression for the largest amount of cotransfected JDP-2 (Fig. 9). The level of reporter gene expression induced by RU486 in the presence of cotransfected JDP-2 was ~10% of that induced by R5020 in the presence of JDP-2 (data not shown) (compare Fig. 5). However, this effect of JDP-2 on PR occupied by RU486 is substantial, as the level of gene transactivation is equal to that induced by the progestin agonist in the absence of exogenous JDP-2 (Fig. 9).

JDP-2 interacts with other general coactivators and forms a ternary complex with PR on DNA. Because JDP-2 interacts with the DBD of PR, it was important to determine whether it might have an influence on PR-DNA binding. As detected by EMSA with a consensus PRE oligonucleotide, over a wide range of concentrations and ratios, JDP-2 with PR had minimal effect on specific PR-DNA binding activity (data not shown). JDP-2 neither inhibited nor enhanced the formation of the PR-PRE complex. However, addition of JDP-2 to the DNA binding reaction resulted in the formation of a ternary PR–JDP-2–DNA complex, as evidenced by the supershift with antibodies to either PR or JDP-2 (Fig. 10A). JDP-2 alone at the doses used did not bind tightly to the PRE probe, suggesting that its presence in the PR-DNA complex is primarily through protein interaction. These results support the conclusion that JDP-2 is capable of interacting with PR bound to DNA without significantly affecting PR-DNA binding.

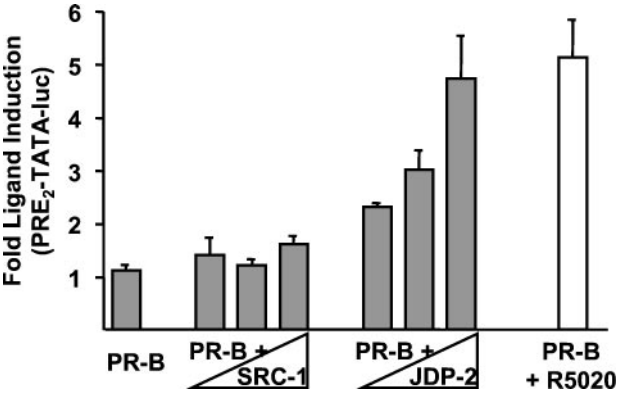

FIG. 9. JDP-2 potentiates the partial agonist activity of RU486. Cos-1 cells were transfected with phPR-B (1.5 ng) and PRE_2-TATA-luc (200 ng) in the presence or absence of equimolar doses of pCR3.1-SRC-1 (50 to 200 ng) or pCR3.1-JDP-2 (34 to 137 ng). Cells were treated with vehicle, 10 nM RU486 (dark bars), or 10 nM R5020 (open bar) for the last 24 h of transfection. Several fold hormone induction values were calculated as for Fig. 5, and values are averages ± SEM of three independent experiments.

JDP-2 does not contain an autonomous transcription activation domain (3). Therefore, we hypothesized that its ability to stimulate PR-mediated transactivation is due to JDP-2 recruitment of other coactivators that possess activation domains. Other coactivators, including CBP, NCoA-62, SRC-1, GRIP1 (SRC-2), and pCAF, were analyzed for their ability to bind to JDP-2 independent of PR. As shown by GST pull-down assay, CBP, NCoA-62, and pCAF all bound efficiently and specifically to JDP-2 (Fig. 10B). In contrast, neither SRC-1 nor SRC-2 (GRIP-1) showed any specific binding to JDP-2 (Fig. 10B). The efficiency of JDP-2 interaction with CBP, NCoA-62, and pCAF was equal to that of JDP-2 interaction with c-Jun (Fig. 10B).

DISCUSSION

The amino-terminal domain of human PR is responsible for isoform-specific activities as well as cell- and promoter-specific responses to progestins, yet the interacting proteins that mediate activities of the N-terminal domain have not been well defined. Here we discovered that an AP-1 family member, JDP-2, initially described as a repressor of c-Jun transactivation of AP-1 response elements (3), also has functional activity as a coactivator of progesterone-dependent PR-mediated gene transcription. JDP-2 can stimulate independently the activity of ligand-inducible AF-2 in the C terminus, as well as of constitutive N-terminal AF-1. However, JDP-2 has dramatically stronger activity with AF-1. Although the mechanism by which

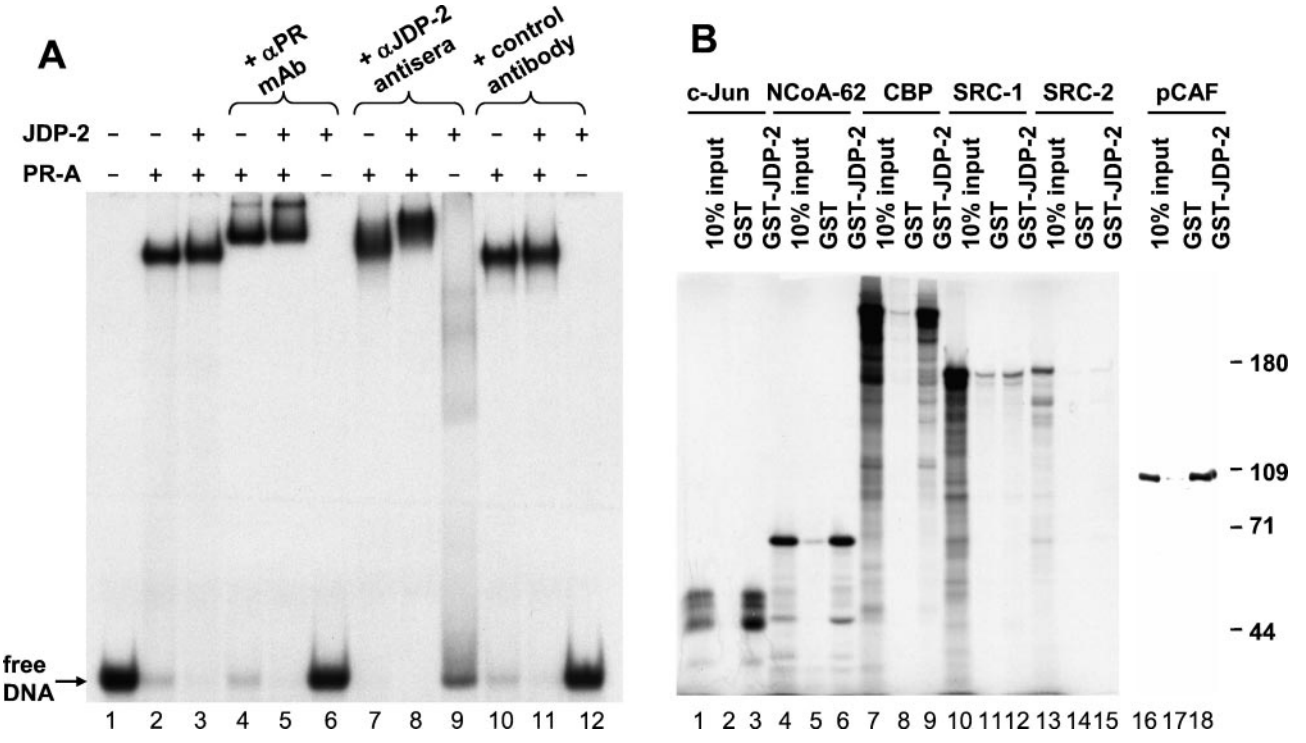

FIG. 10. JDP-2 forms a ternary complex with PR on DNA and interacts with general coactivators but not with SRCs (p160s). (A) Purified recombinant PR-A (30 nM) bound to R5020 and purified JDP-2 (200 ng) were incubated alone or together with 0.3 ng of a 32-bp synthetic [^{32}P]PRE oligonucleotide probe for 1 h on ice. To supershift PR–DNA–JDP-2 complexes, 2 μg of the PR MAb, 4 μg of JDP-2 antiserum, or an unrelated control antibody (as indicated above appropriate lanes) were added for the final 30 min of incubation with DNA. Samples were separated on 5% nondenaturing polyacrylamide gels, followed by drying of the gels and detection of protein-DNA complexes by autoradiography. (B) c-Jun, NCoA-62, CBP, and SRC-1 and SRC-2 (GRIP-1) were transcribed and translated in vitro using rabbit reticulocyte lysate supplemented with [^{35}S]methionine. pCAF with an N-terminal FLAG tag was expressed in Sf9 cells. Labeled coactivators or FLAG-pCAF were incubated with GST or GST–JDP-2 immobilized to glutathione-Sepharose. Resins were washed in a buffer containing 100 mM NaCl, and bound proteins were eluted and detected either by autoradiography or by Western blotting with an antibody to the FLAG tag (pCAF). Lanes contain 10% input or coactivator interaction with GST or GST–JDP-2 as indicated.

JDP-2 functions as a coactivator of PR AF-1 is not completely understood, our results provide initial insights. JDP-2 interacts directly with PR through the DBD, and this protein interaction appears to be essential for JDP-2 stimulation of PR transactivation, as evidenced by a loss of JDP-2 activity on chimeric receptors consisting of the Gal4 DBD in place of the PR DBD (Fig. 8). Although the PR DBD is required for a functional response to JDP-2, it is not sufficient. We also showed by use of receptor chimeras and domains of PR (Figs. 7 and 8) that the PR DBD, together with receptor's own AF-1, is required for coactivation by JDP-2. JDP-2 lacks a transcription activation domain; however, we show that it interacts with other general coactivators such as CBP and pCAF that do have intrinsic activation domains and HAT activity (Fig. 10B). These results suggest that JDP-2 associates with the DBD as a larger coactivator complex.

The SRC family of nuclear receptor coactivators interacts with AF-2 in the LBD primarily in a ligand-dependent manner through a specific LXXLL motif. SRCs also have the ability to interact with and potentiate N-terminal AF-1 through a secondary interaction site separate from the LXXLL motifs (1, 44, 61). Our results provide several lines of evidence that SRC-1 is not involved in JDP-2 stimulation of AF-1. JDP-2 had substantially higher effect on AF-1 than SRC-1; the two proteins did not act synergistically on AF-1; a protein-protein interaction between JDP-2 and SRCs was not detected; and JDP-2, but not SRC-1, potentiated the partial agonist activity of RU486. The difference in the effects of JDP-2 and SRC-1 on PR occupied by RU486 may be significant. Partial agonist effects of RU486 have been shown to require an intact AF-1 (41), suggesting that JDP-2 and SRC-1 do not affect AF-1 in the same way. JDP-2 and SRC-1, however, did stimulate ligand-dependent AF-2 activity synergistically, suggesting that the weak effect of JDP-2 on AF-2 is through association with a component of the SRC-1 coactivator complex assembled on AF-2. Our results are consistent with a model whereby JDP-2, through direct association with the DBD, functions as a docking factor to recruit or stabilize other coactivator interactions with AF-1 in the N terminus that lies adjacent to the DBD. Whether CBP/pCAF or other unknown coactivators are recruited by JDP-2 in vivo as a result of this function remains to be determined.

There is precedent for coactivator recruitment through docking factors that interact with the DBD of nuclear receptors. Similar to JDP-2, the cold-induced coactivator of PPARγ, PGC-1, has minimal transcription activity. Interaction with the DBD of PPARγ induces a conformational change in PGC-1 that enables its interaction with other coactivators such as SRC-1 and p300/CBP. Thus, PGC-1, through association with the DBD, provides an alternate pathway to AF-2 for recruiting

coactivators that possess HAT and transcription activity (48). The histone acetylase pCAF was initially thought to be recruited by nuclear receptors indirectly, through its association with CBP as part of the SRC-1 coactivator complex assembled at AF-2. However, pCAF has also been shown to directly associate with the DBD of several nuclear receptors independent of p300/CBP (7, 30). With retinoid X receptor/retinoic acid receptor (RAR) heterodimers, this direct pCAF-DBD interaction potentiates ligand-dependent transcription activation, and pCAF is believed to associate with the DBD as a larger human ADA2 homologue complex (7).

Direct protein interaction between PR and JDP-2 was mapped to the DBD of PR and the bZIP region of JDP-2. Other nuclear hormone receptors, including RAR, GR, ERα, and AR, have also been shown to physically and functionally interact with another bZIP protein, the C/EBP (11, 51, 52). ER and RAR were reported to repress (51, 52) whereas GR potentiated (11) C/EBPβ-mediated transcription on promoters that lacked steroid response elements. Direct protein interactions mapped to the DBDs and hinge regions of receptors and to the bZIP region of C/EBP (11, 52). Analogous to our studies with PR and JDP-2, potentiation of C/EBPβ activity by GR required AF-2 in the LBD, whereas physical protein interaction required the DBD (11), suggesting that the DBD and an activation domain are required together for functional interactions. More recently, progesterone activation of the prolactin promoter in decidual tissue was shown to be mediated by physical interaction of PR with C/EBP on the prolactin promoter (14). These results taken together suggest that protein interaction with bZIP proteins is a common property of nuclear receptors. The specific motifs in the nuclear receptor DBD and the bZIP domain that mediate these interactions remain to be determined.

In addition to a direct protein interaction between PR and JDP-2 in vitro, a physical association between the two proteins was detected in mammalian cells by coimmunoprecipitation assay. Because JDP-2 is expressed at low levels in the cell lines used in these experiments, we initially performed coimmunoprecipitation of exogenously expressed PR and JDP-2 as a means to increase cellular levels of the proteins and the sensitivity of the assay (Fig. 4A and B). However, coimmunoprecipitation of endogenous JDP-2 and PR from T47D breast cancer cells was also possible (Fig. 4C). Using a ChIP assay, endogenous JDP-2 and PR were also observed to be recruited by the promoter of a progestin inducible promoter (MMTV) in vivo in the context of chromatin (Fig. 4D). JDP-2 protein interaction with PR was ligand independent both in vitro (pull-down assay) and in vivo (coimmunoprecipitation assay), yet JDP-2 stimulation of PR-mediated gene transcription was highly ligand dependent. The reason for this apparent discrepancy is not known. Because PR binding to DNA is hormone dependent, JDP-2 could be recruited to specific promoters through constitutive interaction with PR. In support of this model, ChIP experiments showed a strong hormone-dependent recruitment of PR by the MMTV promoter as well as a less pronounced but significant increased recruitment of JDP-2 in response to hormone (Fig. 4D).

Because JDP-2 interacts with PR through the DBD, it was important to determine whether it can form a ternary complex with PR on DNA and whether the presence of JDP-2 might in part enhance receptor transcription activity by stabilizing PR-DNA binding. In EMSAs, JDP-2 had little or no influence on PR-DNA binding. JDP-2 was able to form a ternary complex with PR and DNA, as evidenced by supershift of DNA complexes by either PR or JDP-2 specific antibodies in the presence of both PR and JDP-2 proteins. It was difficult to detect a change in the mobility of the PR-DNA complex by the addition of JDP-2 in the absence of antibody, presumably because JDP-2 is a much smaller protein (21 kDa) than PR (80 to 100 kDa) (Fig. 10A). Nonetheless, we did observe slightly reduced mobility of PR-PRE complexes as the result of the addition of JDP-2 to EMSAs, although this finding was not consistent in every experiment (Fig. 10A). These results suggest that JDP-2 does not affect PR transactivation in cells at the level of facilitating PR-DNA binding and is capable of interacting with PR bound to DNA.

AF-1 of PR can function as an autonomous activation domain when linked to the heterologous Gal4 DBD, and the minimum boundaries required for AF-1 activity (aa 456 to 546) were defined by truncation mutations of the Gal4DBD–AF-1 construct (42). Although JDP-2 increased transactivation mediated by a minimal AF-1–DBD construct (using the definition of AF-1 as reported by Meyer et al. [42]), the magnitude of the effect was less than that observed with the whole PR-A N-terminal domain linked to the DBD. This suggests that AF-1 requires additional sequences N-terminal to aa 456 for maximal activity and response to JDP-2. Alternatively, AF-1 may be composed of multiple discontiguous sequences in the N-terminal domain that together form a protein recognition motif. While biophysical studies have indicated the N domain of steroid receptors, including PR (4), exists in a nonglobular extended conformation, proteins interacting with one or more elements of AF-1 could induce secondary structures required for maximal AF-1 activity. Further work will be necessary to more precisely define N-terminal sequences required for coactivation by JDP-2 and for full AF-1 activity.

Of the nuclear receptors tested, JDP-2 exerted preferential activity on PR. Because the N-terminal domain is the least conserved region among nuclear receptors, this preference for PR is consistent with the model of JDP-2 acting predominantly through AF-1. One might expect an AF-1 coactivator to be fairly receptor specific. In this regard, JDP-2 also exhibited weak activity on GR. Of the other receptors tested, GR is most closely related to PR. The lack of activity of JDP-2 on transcription properties of other nuclear receptors does not appear to be due to an inability of JDP-2 to form a protein interaction. In the GST pull-down assay, JDP-2 interaction was detected with DBDs of the other nuclear receptors tested (data not shown). These results further highlight the finding that the DBD, together with a specific activation domain, is required for functional response to JDP-2. JDP-2 has been reported to be expressed in a broad range of cells and tissues (3, 31). We now show that JDP-2 is also expressed in PR target tissues and in progesterone-responsive breast cancer cells (T47D), suggesting that it has a physiological role in PR-specific functional responses. Whether JDP-2 is regulated in progesterone target cells and to what extent cellular levels of JDP-2 might influence PR activity are important questions. The fact that overexpression of JDP-2 strongly potentiated the partial agonist of RU486 suggests that cellular levels of JDP-2 can significantly

influence the pharmacology of PR antagonists. The PR antagonist ZK98299 is a pure antagonist and represses PR transactivation in most contexts previously assayed. JDP-2 had no effect on ZK98299-occupied receptor (data not shown), suggesting that its potentiation of RU486 partial agonist activity is not counter to previously described activities of PR. Another bZIP protein, L7/SPA, was previously reported to enhance transactivation by RU486-occupied PR (29). However, L7/SPA appears to work by a different mechanism than JDP-2, as it had no effect on agonist-occupied PR and interaction mapped to the hinge region rather than the DBD of PR (29). The only other PR coactivator shown to increase transactivation through an N-terminal AF-1 mechanism is steroid receptor RNA coactivator (SRA) (34). SRA is an RNA transcript shown to exist as a component of a larger protein complex that contains SRC-1. The relationship, if any, between SRA and JDP-2 is unknown. SRA was reported to be selective for enhancing activity of steroid receptors, so it is not preferential for PR, and whether SRA influences the partial agonist activities of antagonists was not reported (34).

JDP-2 was initially defined as a repressor of bZIP transcription factors, including Jun, ATF-2, and C/EBPγ (3, 12, 27, 31, 46). JDP-2 possesses the consensus leucine zipper and basic residues required for dimerization and DNA binding, but lacks the C-terminal activation domain (Fig. 1). JDP-2-mediated repression of c-Jun is thought to occur in part through heterodimerization with Jun on DNA and through preventing the formation of active Fos-Jun heterodimers and similarly through formation of heterodimers on DNA with other bZIP proteins. However, JDP-2 also contains an active repression domain (46). Mapping of JDP-2 functional domains by fusing regions to the Gal4 DBD has indicated that the C-terminal domain possesses most of the repression activity of JDP-2 (A. Aronheim, unpublished data). An interesting and potentially important question is how JDP-2 can function as a repressor of Jun and a coactivator of PR. Our analysis of different domains of JDP-2 for activity on PR showed that the C-terminal domain was dispensable (Fig. 3B), whereas the N-terminal domain together with the bZIP region was required for stimulation of PR transcription activity. Taken together, these data suggest that JDP-2 uses different interaction surfaces in its roles as coactivator and as corepressor: namely, the N domain for coactivation of PR and the C domain for corepression of bZIP protein. There is precedent for other nuclear receptor interacting proteins that can function as coactivators or corepressors under different conditions. FKHR (forkhead homologue of rhabdomyosarcoma), TIF1, and NSDI can either enhance or repress nuclear receptor transcriptional activity depending on the cell and promoter context (26, 35, 64). The Ski oncogene interacting protein (Skip), possibly important in cell transformation mediated by Ski (16), can also repress or coactivate nuclear receptors and other transcription factors dependent on cellular context (5, 65, 66).

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ADDENDUM IN PROOF

After submission of our report, another low-molecular-weight bZIP protein was reported to interact with several nuclear receptors through their DNA binding domains and to function as a potent coactivator by mechanisms distinct from that of ligand-dependent AF-2 coactivators such as SRCs (L. Ko, G. R. Cardona, A. Henrion-Caude, and W. W. Chin, Mol. Cell. Biol. **22:**357–369, 2002).

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