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The Neuroendocrine-Derived Peptide Parathyroid Hormone–Related Protein Promotes Prostate Cancer Cell Growth by Stabilizing the Androgen Receptor

John DaSilva, Daniel Gioeli, Michael J. Weber, and Sarah J. Parsons

Department of Microbiology and Cancer Center, University of Virginia Health System, Charlottesville, Virginia

Abstract

During progression to an androgen-independent state following androgen ablation therapy, prostate cancer cells continue to express the androgen receptor (AR) and androgen-regulated genes, indicating that AR is critical for the proliferation of hormone-refractory prostate cancer cells. Multiple mechanisms have been proposed for the development of AR-dependent hormone-refractory disease, including changes in expression of AR coregulatory proteins, AR mutation, growth factor–mediated activation of AR, and AR protein up-regulation. The most prominent of these progressive changes is the up-regulation of AR that occurs in >90% of prostate cancers. A common feature of the most aggressive hormone-refractory prostate cancers is the accumulation of cells with neuroendocrine characteristics that produce paracrine factors and may provide a novel mechanism for the regulation of AR during advanced stages of the disease. In this study, we show that neuroendocrine-derived parathyroid hormone–related protein (PTHrP)–mediated signaling through the epidermal growth factor receptor (EGFR) and Src pathways contributes to the phenotype of advanced prostate cancer by reducing AR protein turnover. PTHrP-induced accumulation of AR depended on the activity of Src and EGFR and consequent phosphorylation of the AR on Tyr^{534}. PTHrP-induced tyrosine phosphorylation of AR resulted in reduced AR ubiquitination and interaction with the ubiquitin ligase COOH terminus of Hsp70-interacting protein. These events result in increased accumulation of AR and thus enhanced growth of prostate cancer cells at low levels of androgen. [Cancer Res 2009;69(18):7402–11]

Introduction

Expression of the androgen receptor (AR) plays an integral role in the progression of prostatic adenocarcinoma to hormone-refractory disease (1, 2). Several mechanisms have been described to account for the development of AR-dependent, androgen-refractory prostate cancer, including AR mutational activation, coactivator overexpression, and activation via cross-talk with growth factor signaling pathways. The most frequent mechanism, however, involves increases in AR protein levels.

Increases in AR are both necessary and sufficient to convert prostate cancer cells from a hormone-sensitive to a hormone-refractory state (3). This finding is consistent with reports showing that high levels of AR are associated with aggressive clinicopathologic features and with decreased recurrence-free survival in prostate cancer patients treated with radical prostatectomy (4). In addition, immunohistochemical studies have shown that the AR is expressed in essentially all human prostate cancers, including those that have become hormone refractory following hormone ablation therapy, and that the level of AR expression is often increased relative to untreated tumors (5, 6).

One explanation for the elevated levels of AR is gene amplification, reported to occur in ~22% of advanced prostate tumors (7). However, increased levels of AR observed in the majority of prostate cancers may also result from increased AR protein stability. As seen in recurrent CWR22 and LNCAP xenograft tumors (8, 9), increases in AR are sufficient to allow continued androgen signaling under conditions of low circulating ligand. Chen and colleagues (3) have shown that increasing the concentration of AR in prostate cancer cells using an AR-expressing lentivirus reduces the latency period for the development of LNCaP and LAPC4 xenograft tumors in castrate mice, supporting the hypothesis that increased AR protein promotes growth and survival of prostate tumors in low levels of androgen.

Another mechanism postulated to promote the progression of androgen-refractory prostate cancer is the appearance of cells with a neuroendocrine phenotype. Neuroendocrine-like cells are more prevalent in androgen-refractory disease, occurring in 30% to 100% of tumors studied (10, 11). The low proliferative capacity of neuroendocrine cells allows them to resist treatment with most chemotherapeutic agents as well as endocrine and radiation treatments (12, 13). Neuroendocrine-like cells are thought to provide growth and survival signals to surrounding tumor cells, promoting the progression of hormone-refractory prostate cancer (14, 15). This hypothesis is supported by work showing that neuroendocrine-like cells enhance the growth of LNCaP xenografts, with the greatest effects seen under conditions of androgen deprivation (16, 17).

Parathyroid hormone–related protein (PTHrP) is one of the secreted products of prostatic neuroendocrine cells, and its expression in the human prostate is a manifestation of abnormal growth regulation. In culture, PTHrP cooperates with low levels of androgen to promote growth of androgen-dependent prostate cancer cells, but the molecular mechanisms responsible for these effects remain unclear (18). In particular, it is not known whether the effects of PTHrP on growth are dependent on the AR.

In the present study, we describe a novel mechanism by which neuroendocrine-derived factors, such as PTHrP, provide a proliferative advantage to prostate cancer cells. Our studies reveal that LNCaP cells at low androgen concentration proliferated more rapidly when stimulated with PTHrP. Under these conditions, steady-state levels of AR protein were increased and this increase, like PTHrP-induced proliferation, required the activation of

Requests for reprints: Sarah J. Parsons, Department of Microbiology, University of Virginia Health System, P. O. Box 800734, Charlottesville, VA 22908-0734. Phone: 434-924-2352; Fax: 434-982-0689; E-mail: sap@virginia.edu.

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epidermal growth factor receptor (EGFR) or Src and the subsequent phosphorylation of the AR on Tyr^{534}. We found that targeting of AR for proteasomal-dependent degradation via the chaperone-associated ubiquitin ligase *COOH terminus of Hsp70-interacting protein* (CHIP) was decreased, resulting in the accumulation of AR in response to PTHrP. The phosphorylation of AR on Tyr^{534} reduced the interaction of AR with CHIP. Together, these findings show that neuroendocrine-derived PTHrP stimulation of cancer cells results in the up-regulation of AR protein, thereby promoting growth under low androgen conditions and progression of hormone-refractory disease.

Materials and Methods

Cell culture, transfections, and reagents. LNCaP cells were maintained in T-medium with 5% (v/v) fetal bovine serum (FBS; Invitrogen) in a humidified chamber at 37°C with 5% CO_2. Transfections were performed using TransIT-Prostate Transfection kit (Mirus) according to the manufacturer's instructions. The plasmid encoding FLAG-tagged AR has been previously described (19). The FLAG-AR-Y534F expression vector was constructed by subcloning AR-Y534F (gift of Dr. Young E. Whang, University of North Carolina, Chapel Hill, NC) into pCDNA3.1-FLAG plasmid (20). For experiments with cycloheximide (Calbiochem), cells were treated with cycloheximide (10 μg/mL) throughout the period of R1881 and PTHrP stimulation.

Antibodies and reagents were obtained from the following sources: anti-AR PG-21 (Upstate); anti-FLAG antibody, M2 (Sigma); anti–phospho-Src (Tyr^{418}), anti-Src, anti–phospho-EGFR (Tyr^{992}), and anti-EGFR (Biosource); anti-PTH/PTHrP receptor, anti-ubiquitin, and anti-CHIP (Santa Cruz Biotechnology); anti-tubulin (Calbiochem); anti-Hsp90 (StressGen); goat anti-rabbit and goat anti-mouse antibodies (GE Healthcare); Alamar Blue (Biosource); PTHrP (1–34 amino acids), PP2, AG1478 (Calbiochem); and PTHrP receptor antagonist ($Nle^{8,18}$, Tyr^{34})-pTH(3-34)amide (Bachem).

The anti–phospho-Tyr^{534} antibody was raised against a peptide spanning the Tyr^{534} phosphorylation site in human AR by standard methods. The peptide (DSYSGPpYGDMRLETC) was synthesized with phosphotyrosine and an NH_2-terminal cysteine (Anaspec), coupled to keyhole limpet hemocyanin, and used for antibody production in rabbits (Cocalico

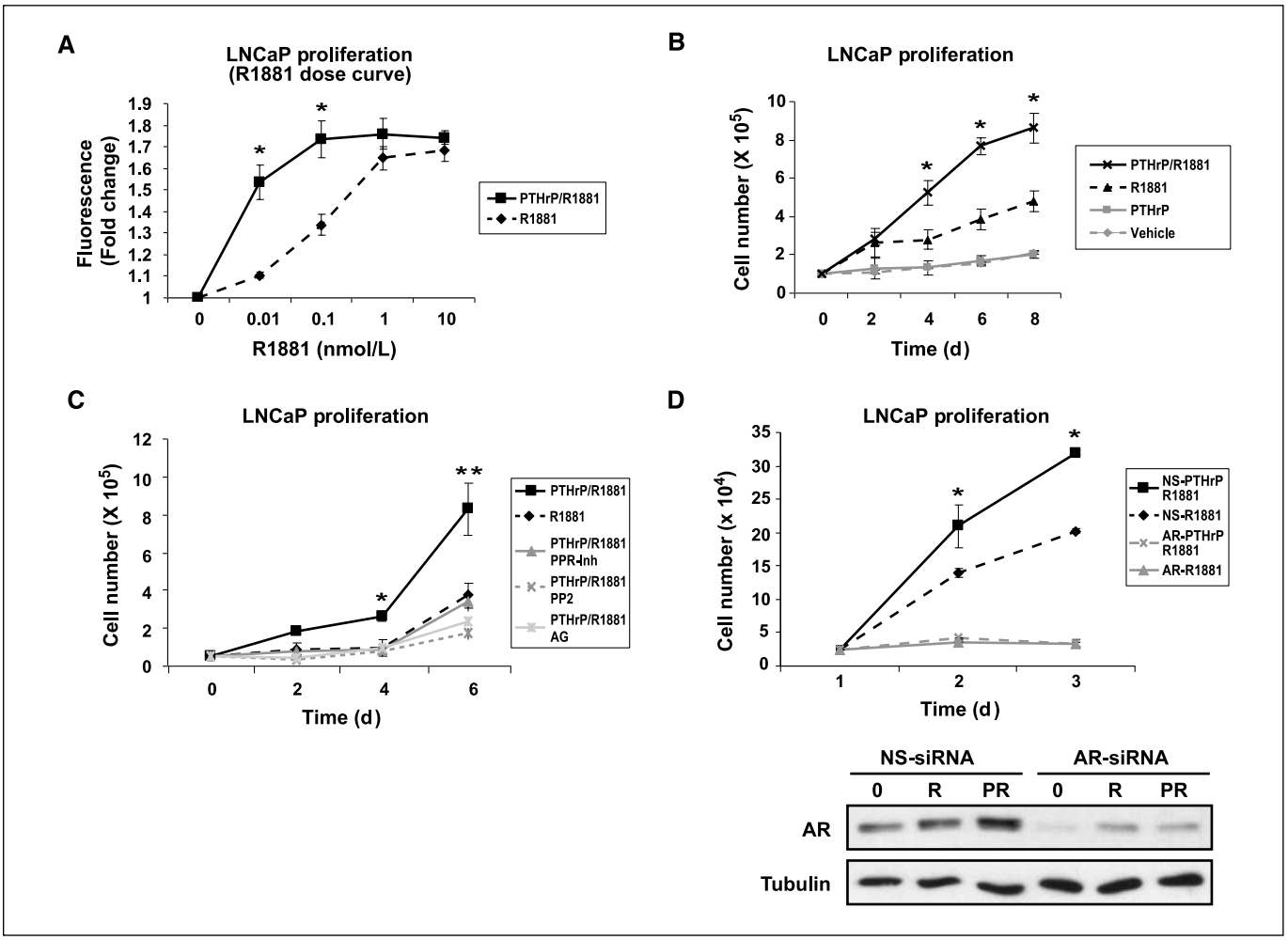

Figure 1. PTHrP enhances proliferation of LNCaP cells. *A,* growth of cells, maintained for 2 d in the indicated concentration of R1881 and 1% CSS with or without PTHrP (10 nmol/L), was monitored with the Alamar Blue assay. *Points,* mean fluorescence of three independent experiments; *bars,* SE. *Asterisks,* significant difference of PTHrP/R1881 compared with R1881 treatment based on a Student's *t* test. *, $P < 0.05$. *B,* 5×10^4 cells were grown for 24 h in T-medium containing 5% FBS, transferred to RPMI 1640 supplemented with 1% CSS, and treated with R1881 (0.1 nmol/L), PTHrP (10 nmol/L), or both. Cells were counted by hemacytometer. *Points,* mean cell number of three independent experiments; *bars,* SE. *, $P < 0.05$. *C,* PTHrP/R1881-stimulated cells were treated with inhibitors of the PTHrP receptor (*PPRInh*), Src (PP2), EGFR (AG1478), or vehicle (DMSO). Cell number was determined by hemacytometer counting. *Points,* mean cell number of three experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. *D,* cells were transfected with either nonspecific siRNA (*NS*) or siRNA targeting the AR transcript (*AR*). Two days after transfection, the cells were treated as in *B*. Cell number was determined by hemacytometer counting. *Bottom,* immunoblotting of cell lysates was performed to confirm knockdown of AR. *Points,* mean of three experiments; *bars,* SE. *, $P < 0.05$.

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Biologicals). Antibody titers were monitored by immunoblotting, and the terminal bleeds were affinity purified using Sepharose-immobilized peptide. The specificity of the antibody was verified by immunoblot analysis against wild-type (wt) and Y543C mutant AR. The increase in immunoreactivity observed with the phospho-Tyr^{534} antibody in response to EGF was similar to that reported in published studies (21).

Cell proliferation assays. Growth of cells in a 96-well plate format (2,500 per well), maintained for 2 d in various concentrations of R1881 and 1% charcoal-stripped serum (CSS) with or without PTHrP, was monitored with the Alamar Blue assay according to the manufacturer's instructions. Cell numbers were determined by seeding 5×10^4 cells/mL in triplicate onto six-well plates in RPMI 1640 without phenol red, supplemented with 0.1 nmol/L R1881 and 1% CSS. The cells were treated with PTHrP alone or together with PTHrP receptor antagonist (10 nmol/L), PP2 (10 μmol/L), AG1478 (5 μmol/L), or DMSO control and counted using a hemacytometer.

Western blotting and immunoprecipitation. LNCaP cells were serum starved overnight in RPMI 1640 and stimulated with R1881 (0.1 nmol/L) or PTHrP (10 nmol/L) for the indicated times. The cells were lysed in radioimmunoprecipitation assay buffer [137 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 10% glycerol (v/v), 1% Triton X-100, 0.5% (w/s) deoxycholate, 0.1% (w/s) SDS, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, protease inhibitors]. The lysate protein concentration was determined using the bicinchoninic acid–containing assay (Pierce).

Endogenous AR was immunoprecipitated from LNCaP cells with 5 μg of anti-human AR per 100-mm dish, and immunoprecipitates were captured on protein A–conjugated Sepharose beads (Roche). FLAG-AR was immunoprecipitated with M2 anti-FLAG antibody coupled to agarose (Sigma). Precipitates were resuspended in sample buffer [10% (v/v) glycerol, 62.5 mmol/L Tris (pH 6.8), 2% (w/v) SDS, 0.01 mg/mL bromphenol blue], resolved by SDS-PAGE, and transferred to nitrocellulose for immunoblotting.

RNA interference. Silencing of Src was performed with Validated Stealth RNAi DuoPak (Invitrogen) according to the manufacturer's instructions. Transfection of Src small interfering RNA (siRNA) was done in LNCaP cells using Oligofectamine (Invitrogen) in Opti-MEM medium. A nonspecific control duplex-XIII (Dharmacon Research) was used as a siRNA silencing control. Maximal knockdown of Src was observed on day 3 after transfection.

Real-time reverse transcription-PCR. Real-time reverse transcription-PCR was performed as previously described (22). Briefly, total RNA was isolated from LNCaP cells treated for 6 h with PTHrP, R1881, or both using RNeasy kit (Qiagen). RNA quantity was determined with RiboGreen RNA assay kit (Invitrogen). First-strand cDNA was produced following the iScript cDNA synthesis protocol (Bio-Rad). cDNA amplification was conducted on an iCycler optical system in the presence of iQ SYBR Green Mastermix (Bio-Rad). The PCR primers used were the following: AR, 5′-CCTGGCTTCCGCAACTTACAC-3′ (forward) and 5′-GGACTTGTGCATGCGGTACTC-3′ (reverse); prostate-specific antigen (PSA), 5′-TGGTGCATTACCGGAAAGTGGATCA-3′ (forward) and 5′-GCTTGAGTCTTGGCCTGGTCATTTC-3′ (reverse); FKBP51, 5′-AGGAGGGAAGAGTCCCAGTG-3′ (forward) and 5′-TGGGAAGCTACTGGTTTTGC-3′ (reverse); NKX3.1, 5′-GCACATATTTGCATGGAAGG-3′ (forward) and 5′-ACAGCGAGTGCATCTTGTTC-3′ (reverse); DKK1, 5′-CCTTGGATGGGTATTCCAGA-3′ (forward) and 5′-CAGTCTGATGACCGGAGACA-3′ (reverse); and GUS, 5′-CCGACTTCTCTGACAACCGACG-3′ (forward) and 5′-AGCCGACAAAATGCCGCAGACG-3′ (reverse).

Statistical analysis. All data were expressed as the mean ± SE from three or more independent experiments. Statistical analysis was performed by Student's t test. Significance was determined with $P < 0.05$.

Results

PTHrP promotes growth of prostate cancer cells in low androgen. The correlation of hormone-refractory prostate cancer with increased neuroendocrine differentiation and up-regulation of neuropeptide signaling, such as PTHrP (23, 24), prompted us to investigate a potential involvement of PTHrP in androgen-dependent prostate cancer cell proliferation. We performed a dose-response study of the effects of the synthetic androgen R1881 alone or R1881 together with recombinant PTHrP (10 nmol/L) on LNCaP cells. After 48 hours, maximal proliferation induced by R1881 alone occurred at 1 nmol/L. However, a significant growth increase was seen when cells were maintained in medium containing 1% CSS supplemented with as little as 0.01 nmol/L R1881 and PTHrP (Fig. 1*A*), indicating that PTHrP reduces the androgen requirement of LNCaP prostate cancer cells for growth. As indicated in Fig. 1*B*, the proliferative advantage provided by PTHrP occurs only in the presence of R1881.

It has previously been documented that EGFR and Src pathways are induced on G protein–coupled receptor (GPCR) activation (25–28). To gain insight into the role of these pathways in PTHrP-induced proliferation, we evaluated effects of EGFR or Src inhibitors on proliferation of the prostate cancer cells. PTHrP-induced proliferation of the cells was completely abolished by treatment with AG1478 or PP2, the EGFR or Src inhibitors, respectively. Similarly, PTHrP-induced LNCaP cell proliferation was abrogated on treatment with PTHrP receptor antagonist (Fig. 1*C*). These observations indicated that transactivation of both EGFR and Src by the PTHrP receptor is necessary for the proliferation of LNCaP cells in response to PTHrP.

Growth of castrate-resistant prostate cancer occurs in low levels of circulating androgens but requires AR (2). Figure 1*D* shows that growth of LNCaP cells in low-androgen and low-serum medium was significantly increased by PTHrP, and this effect could be blocked by AR siRNA. Together, these data suggest that PTHrP is driving AR-dependent cell growth in this experimental model.

PTHrP stabilizes AR protein. We next analyzed effects of the neuropeptide on AR protein levels. Serum-deprived LNCaP cells were treated with R1881 (0.1 nmol/L), PTHrP (10 nmol/L), or both, and AR expression was determined by immunoblotting. Androgen was found to increase the steady-state expression of AR protein as previously reported, but this effect was further enhanced in the presence of PTHrP. Treatment of LNCaP cells with PTHrP alone did not increase the AR protein level, indicating that the effect of PTHrP signaling on AR is dependent on the presence of androgen (Fig. 2*A*). A dose-response analysis of R1881 alone or together with PTHrP showed that AR protein accumulates to a greater extent at low androgen concentrations (0.01–0.1 nmol/L) when PTHrP is present (Fig. 2*B*). Thus, PTHrP signaling leads to increased AR protein under androgen-limiting conditions, suggesting that PTHrP-induced LNCaP cell growth results from its modulation of AR levels.

Significant accumulation of AR protein was observed following treatment of LNCaP cells with PTHrP and R1881 for 6 hours. To determine whether transcriptional up-regulation of the *AR* gene occurs under these conditions, quantitative real-time PCR was used to measure AR mRNA. Induction of AR gene expression was observed on treatment of LNCaP cells with R1881 alone, but no significant change was detected relative to treatment with R1881 and PTHrP (Fig. 2*C*). To ascertain whether PTHrP-induced increases in AR are a result of changes in protein turnover, LNCaP cells were treated with the protein synthesis inhibitor cycloheximide (10 μg/mL). As shown in Fig. 2*D*, treatment with cycloheximide reduced the steady-state level of AR but did not inhibit the stabilizing effect of PTHrP on AR protein, indicating that PTHrP-mediated signaling influences AR levels posttranslationally. Together, these results suggest that the production of

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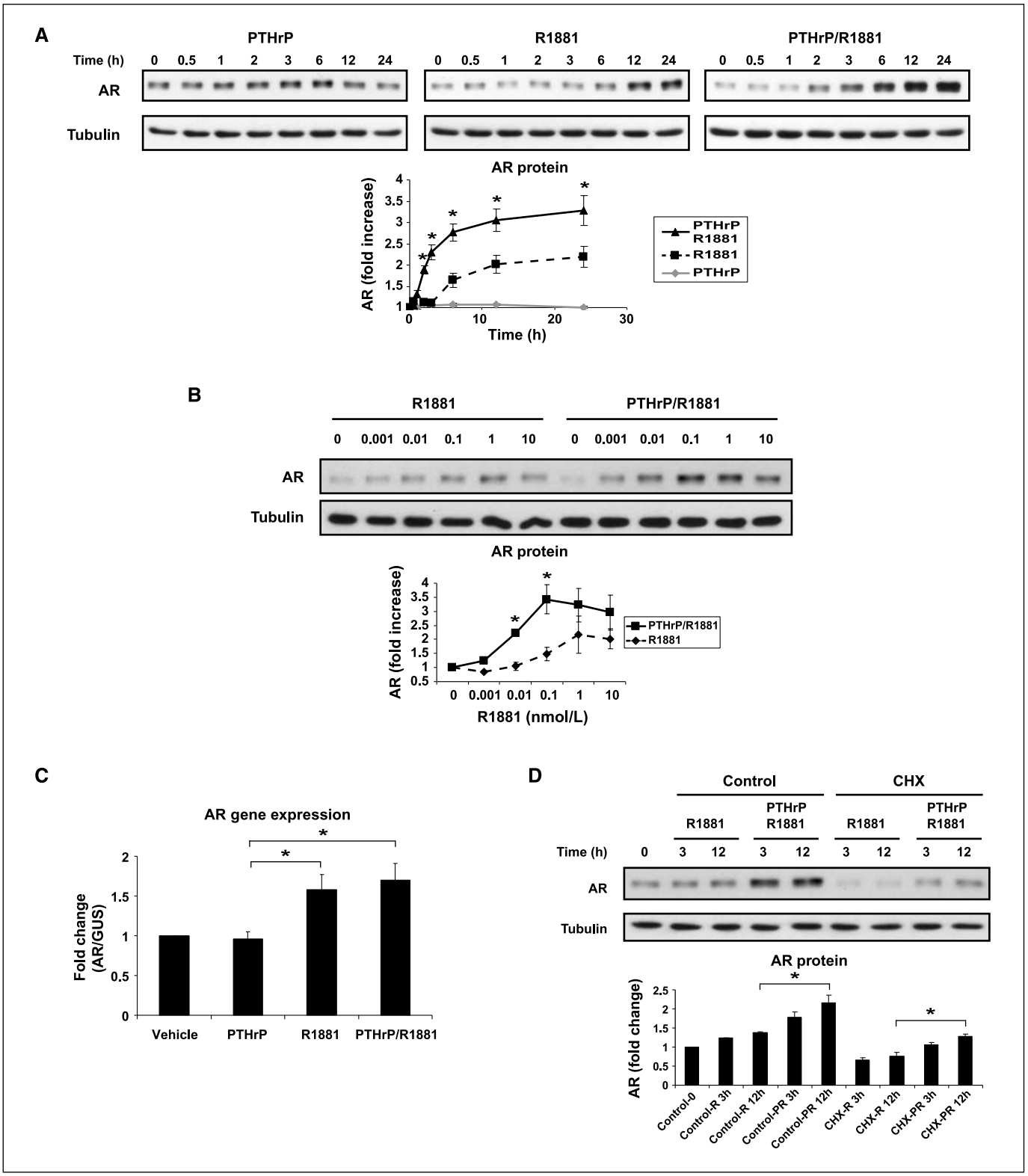

Figure 2. PTHrP stabilizes AR protein. *A,* LNCaP cells were serum starved for 16 h and stimulated for the indicated times with PTHrP (10 nmol/L; *left*), R1881 (0.1 nmol/L; *middle*), or together (*right*). The lysates were analyzed by immunoblotting with anti-AR or anti-tubulin antibodies. Densitometric quantitation of the immunoblot bands was performed. *Points,* mean fold increase of five independent experiments; *bars,* SE. *Asterisks,* significant difference of PTHrP/R1881 compared with R1881 treatment. *, $P < 0.05$. *B,* following serum starvation, LNCaP cells were treated with increasing concentrations of R1881 alone or together with PTHrP (10 nmol/L) as indicated. Lysates were analyzed as described in *A*. Results are representative of three experiments. *, $P < 0.05$. *C,* RNA extracted from LNCaP cells treated for 6 h with PTHrP and R1881, alone or in combination, was analyzed by quantitative real-time PCR using primers specific to AR. Experiments were repeated thrice. The results were normalized to β-glucuronidase reference gene and are expressed as fold change from vehicle treatment. *, $P < 0.05$. *D,* extracts of LNCaP cells stimulated as in *A* and maintained in cycloheximide (*CHX*) or vehicle (DMSO) for the indicated time were immunoblotted as described above. *Columns,* mean AR fold change analyzed after 3- or 12-h treatment; *bars,* SE. *, $P < 0.05$.

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PTHrP by neuroendocrine cells hypersensitizes prostate cancer cells to androgen by decreasing AR turnover and thus increasing the level of AR protein.

PTHrP-induced modulation of AR protein involves EGFR and Src signaling. It is well documented that up-regulation of growth factor signaling correlates with AR activation and progression of prostate cancer (29–31). In addition, stimulation of GPCRs has been shown to result in the transactivation of the EGFR and Src pathways, prompting us to examine whether PTHrP signaling stabilizes AR through the activation of these pathways. To determine whether PTHrP stimulation is capable of activating EGFR in LNCaP cells, phosphorylation of Tyr^{992} was measured by immunoblotting with phosphospecific antibodies. Phosphorylation of Tyr^{992} was increased after 1-hour stimulation with PTHrP in the presence of androgen but was not induced by PTHrP or R1881 alone. Similarly, phosphorylation of the activating Tyr^{418} residue of Src was shown to increase in response to PTHrP and R1881 treatment (Fig. 3*A*).

To determine if the PTHrP-induced activation of EGFR and Src mediates the increase in AR protein, these pathways were blocked pharmacologically. Treatment of LNCaP cells with either AG1478 or PP2 abrogated the AR-stabilizing effect of PTHrP (Fig. 3*B*). Reduction of Src protein in cells transfected with specific siRNA also resulted in a decrease in AR protein level relative to LNCaP cells transfected with control siRNA even in the presence of PTHrP (Fig. 3*C*), further implicating Src in mediating the PTHrP-induced increase in AR protein. Together, these observations suggest that both EGFR and Src signaling are critical to PTHrP-induced regulation of AR.

PTHrP signaling decreases AR interaction with Hsp90 and the E3 ubiquitin ligase CHIP. AR protein turnover is regulated in part by association with chaperones, such as Hsp70 and Hsp90, and occurs via ubiquitin-dependent proteolysis (32, 33). To examine whether the interaction of AR with chaperones is altered in the presence of PTHrP, AR was immunoprecipitated from LNCaP cells deprived of serum and treated with R1881 alone or in combination with PTHrP. As shown in Fig. 4*A*, less Hsp90 was in complex with AR when the cells were treated with R1881 and PTHrP. Analysis of AR ubiquitination following immunoprecipitation also indicated that PTHrP treatment reduced AR ubiquitinated species (Fig. 4*B*). This observation indicated that AR is subject to proteasome-dependent degradation under normal physiologic conditions and that the presence of PTHrP can stabilize the AR protein by interfering with its ubiquitination.

AR is a target of the ubiquitin ligases *Mdm2* or CHIP, leading to degradation by the 26S proteasome (34, 35). Based on the observation that PTHrP reduces the interaction of AR with the Hsp90 chaperone complex, we hypothesized that the reduction in AR ubiquitination may be due to decreased interaction with the CHIP E3 ligase. Analysis of the AR immunoprecipitate showed reduced CHIP protein on treatment with PTHrP (Fig. 4*B*). These data suggest that on PTHrP stimulation the interaction of AR with chaperone-associated ubiquitin ligases and subsequent proteasomal-directed degradation is reduced.

AR tyrosine phosphorylation reduces CHIP interaction and prevents PTHrP-induced accumulation of AR. In previous work, we showed that EGF signaling results in the tyrosine phosphorylation of AR and modulates its interaction with Src (19). To

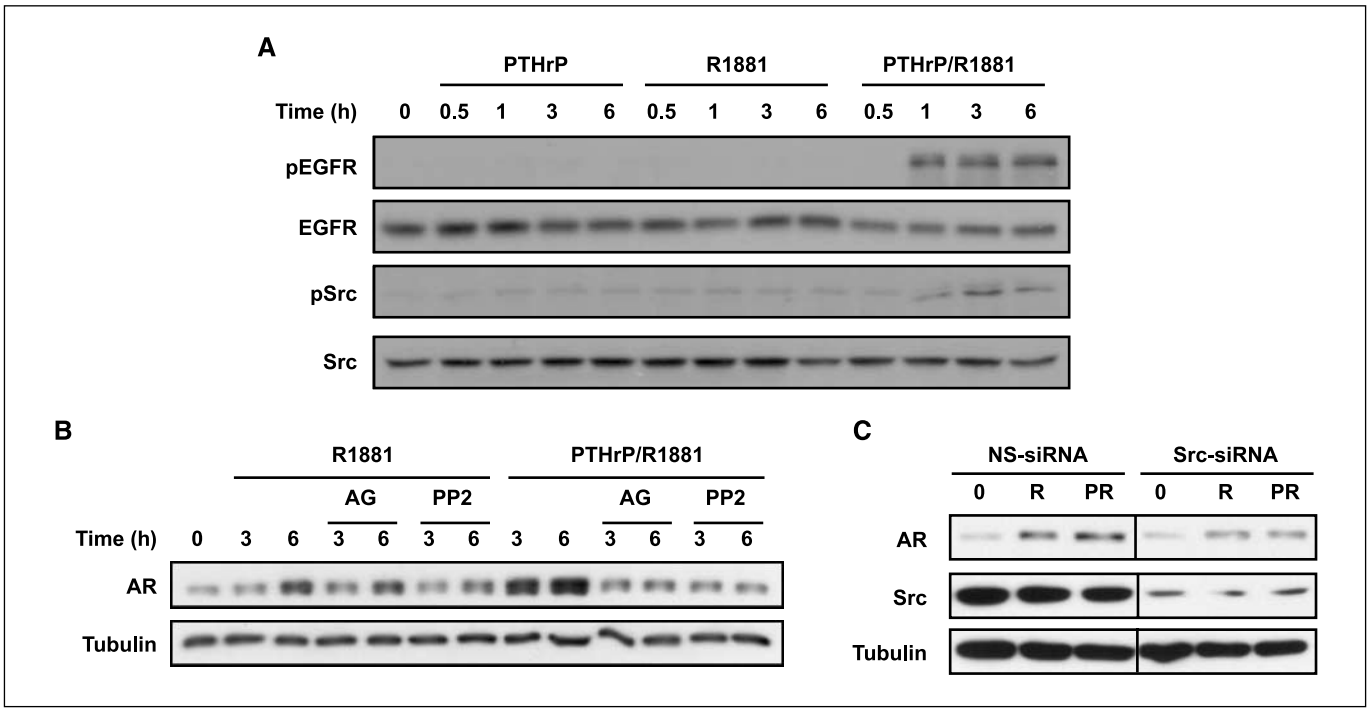

Figure 3. PTHrP modulation of AR protein is mediated by EGFR/Src signaling in LNCaP cells. *A,* LNCaP cells were treated with either R1881 (0.1 nmol/L) alone or together with PTHrP (10 nmol/L) for the indicated time. Immunoblotting was performed with anti–phospho-EGFR (Tyr^{992}), anti-EGFR, anti–phospho-Src (Tyr^{418}), or anti-Src antibodies. *B,* serum-starved LNCaP cells were treated with R1881 (0.1 nmol/L), R1881 together with PTHrP (10 nmol/L), as well as the small molecule EGFR (AG1478, 5 μmol/L) or Src (PP2, 10 μmol/L) inhibitors. Cell extracts were prepared for immunoblotting with anti-AR or anti-tubulin antibodies. *C,* LNCaP cells were transfected with either nonspecific siRNA or siRNA targeting the *Src* gene as described in Materials and Methods. Two days after transfection, the cells were serum starved and treated with R1881 (0.1 nmol/L) alone or together with PTHrP (10 nmol/L) for 12 h. Immunoblotting of cell extracts was performed using anti-AR, anti-Src, or anti-tubulin antibodies.

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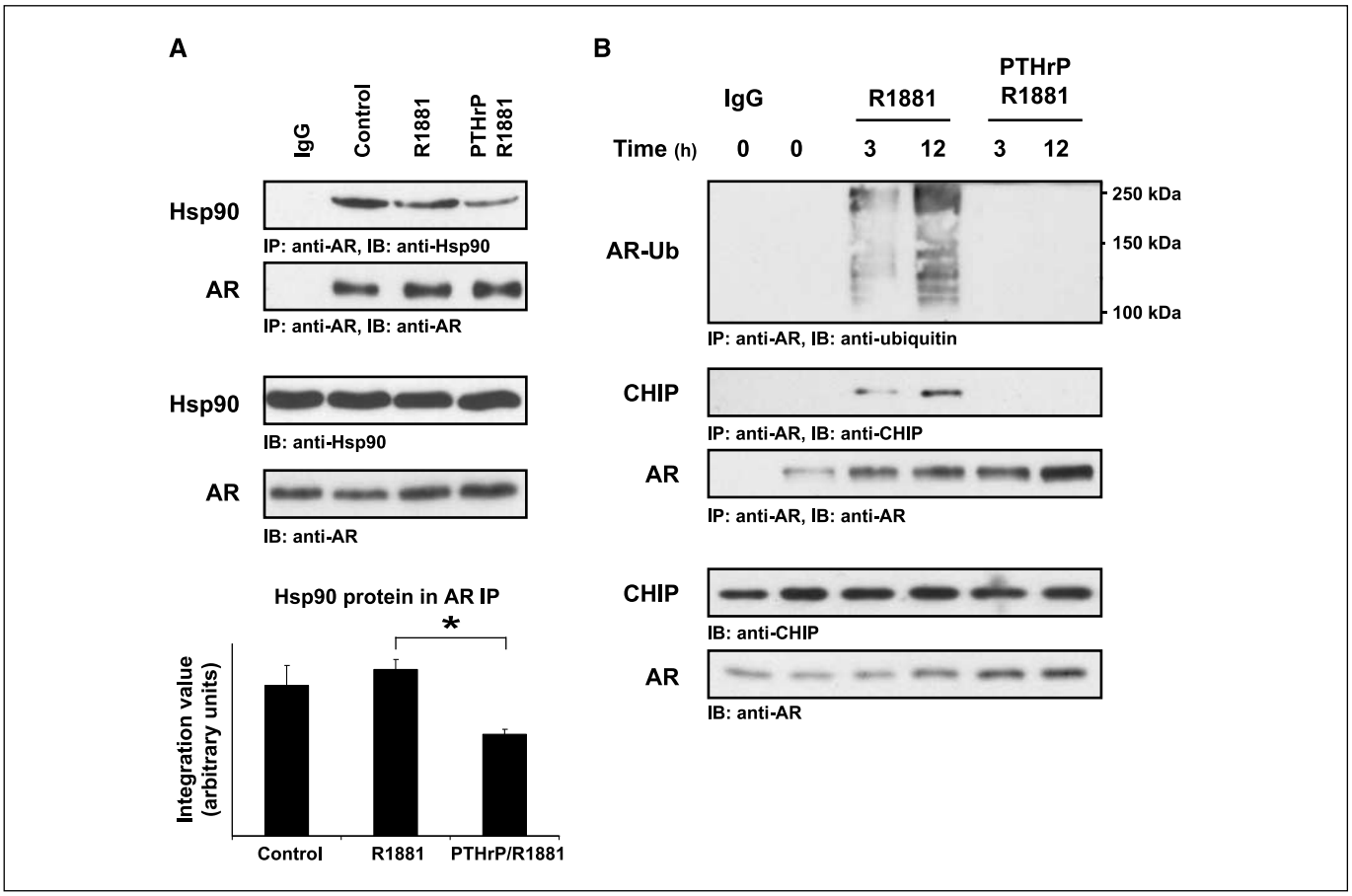

Figure 4. PTHrP signaling decreases AR interaction with Hsp90 and the E3 ubiquitin ligase CHIP. *A,* LNCaP cells were serum starved and stimulated for 12 h with R1881 (0.1 nmol/L) or PTHrP (10 nmol/L) and R1881. *Bottom,* cell extracts were subjected to immunoprecipitation using anti-AR antibodies and immunoblotting with anti-Hsp90 and anti-AR antibodies, and densitometric quantitation of Hsp90 protein coimmunoprecipitated with AR was determined. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$. *B,* LNCaP cells were deprived of serum and stimulated with R1881 or R1881/PTHrP for the indicated times. AR immunoprecipitation of the cell extracts was performed, and an immunoblot was probed with anti-ubiquitin, anti-CHIP, and anti-AR, as indicated. Input levels of AR and CHIP were determined by immunoblotting 5% of the cell extracts.

determine whether PTHrP treatment influences the tyrosine phosphorylation of AR, LNCaP cells were deprived of serum and stimulated with R1881 or R1881 and PTHrP for 6 hours. Cell extracts were subsequently prepared and immunoblotted with phosphospecific antibodies to Tyr^{534}, the primary site of AR tyrosine phosphorylation (21). As shown in Fig. 5*A*, phosphorylation of AR was increased on treatment with R1881 and PTHrP relative to R1881 treatment alone. Similar phosphorylation levels were observed when AR was immunoprecipitated and immunoblotting with anti-phosphotyrosine antibodies was performed (data not shown).

To assess whether the CHIP/AR interaction is modulated by AR tyrosine phosphorylation, LNCaP cells were transiently transfected with either FLAG-AR or FLAG-AR-Y534F. Immunoprecipitation of exogenously expressed FLAG-AR complexes revealed that, similar to endogenous AR, the CHIP interaction is reduced in the presence of PTHrP. However, the phosphorylation-deficient AR-Y534F mutant showed enhanced association with CHIP in response to treatment with R1881 alone or in combination with PTHrP (Fig. 5*B*). This observation suggests that tyrosine phosphorylation of AR is required for decreasing the interaction between CHIP and AR, thus reducing its degradation.

To confirm that the phosphorylation of AR Tyr^{534} plays a role in the PTHrP-induced accumulation of the protein, LNCaP cells ectopically expressing FLAG-AR or FLAG-AR-Y534F were deprived of serum and stimulated with R1881 alone or PTHrP and R1881. Immunoblotting with anti-FLAG antibodies revealed that FLAG-AR, like endogenous AR, accumulated in the presence of PTHrP (Fig. 5*C*). FLAG-AR-Y534F failed to accumulate in response to PTHrP, indicating that the phosphorylation of this site is important for PTHrP-induced stabilization of AR.

PTHrP modulates expression of androgen-regulated genes. We examined AR transactivation in LNCaP cells stimulated with PTHrP, R1881, or both. Quantitative real-time PCR analysis of androgen-regulated genes showed that PTHrP does not significantly modulate NKX3.1 expression in the presence of R1881 but antagonizes R1881 induction of PSA transcription and enhances mRNA levels of FKBP51. Down-regulation of DKK was observed in the presence of R1881 or PTHrP (Fig. 5*D*).

Discussion

Increases in the neuroendocrine status of prostate tumors have been correlated with tumor size and progression to hormone-refractory stages. Existing evidence suggests that neuroendocrine-like cells arise within the tumor on exposure to various differentiation factors, including β-adrenergic agonists, cytokines,

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or long-term androgen ablation (36–38). Although neuroendocrine-like cells are nonmitotic, they produce neuropeptides, such as PTHrP, neurotensin, and bombesin, which are thought to induce proliferative or survival signals to adjacent cancer cells (16, 39). Our data show that PTHrP-mediated signaling enhances the proliferation of prostate cancer cells by an alternative mechanism involving the regulation of AR protein stability.

Androgen ablation therapy reduces the circulating testosterone concentration from 4.5 ng/mL (21 nmol/L) to 0.28 to 1.3 ng/mL (1.3–6 nmol/L), resulting in apoptosis of androgen-dependent

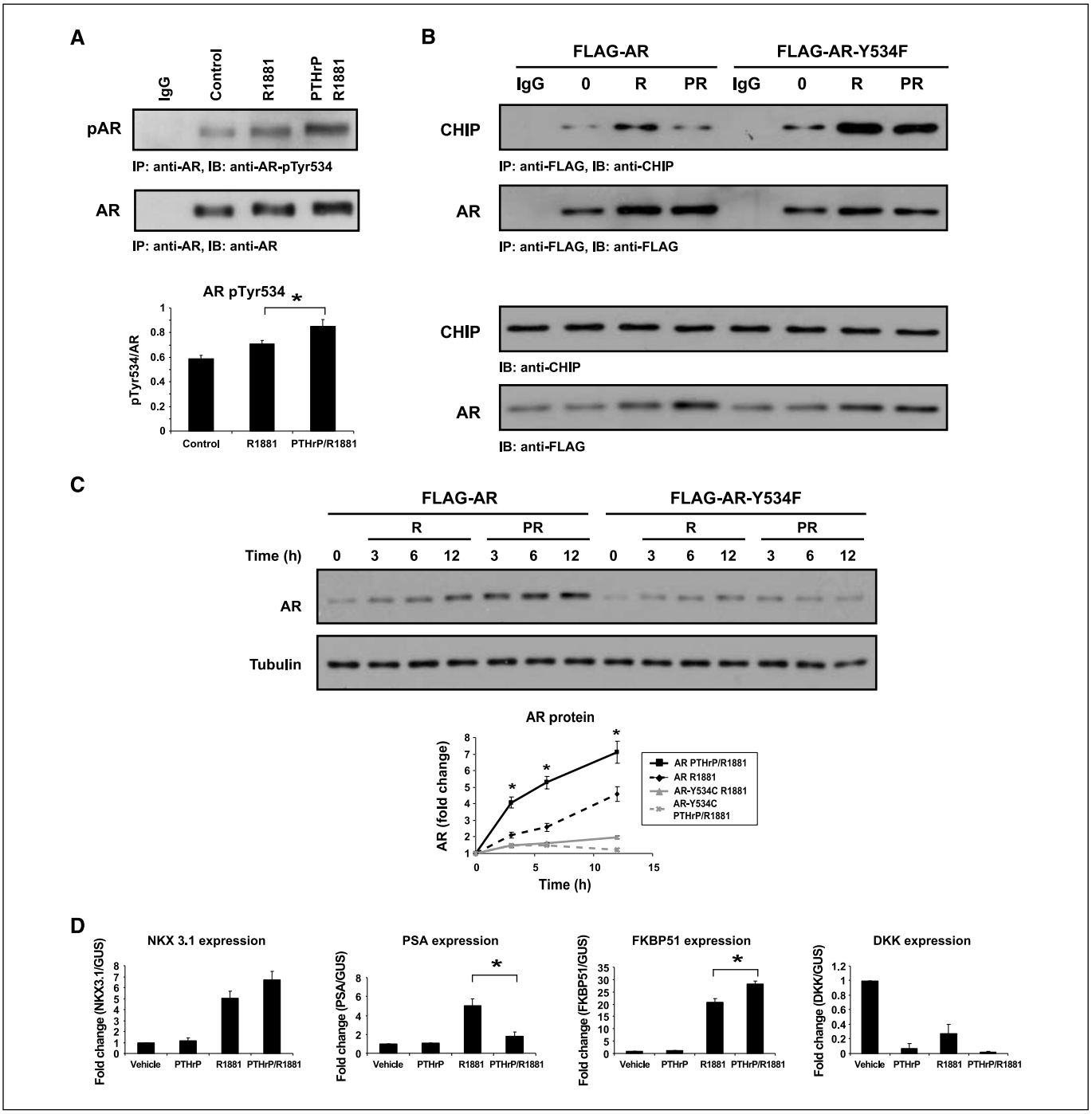

Figure 5. AR tyrosine phosphorylation reduces CHIP interaction and enhances PTHrP-induced accumulation of AR. *A,* LNCaP cells were serum starved and stimulated with vehicle (ethanol), R1881 (0.1 nmol/L), or PTHrP (10 nmol/L) together with R1881 for 12 h. Cell extracts were immunoblotted with anti-AR or anti–phospho-AR (Tyr^{534}). Densitometric quantitation of tyrosine-phosphorylated AR was performed for three independent experiments. *Columns,* mean phospho-Tyr^{534}/AR; *bars,* SE. *, $P < 0.05$. *B,* LNCaP cells were transfected with FLAG-wtAR or FLAG-AR-Y534F and treated as described in *A*. AR immunoprecipitates were probed with anti-CHIP or anti-AR antibodies. Input levels of AR and CHIP were determined by immunoblotting 5% of the cell extracts. *C,* LNCaP cells as in *B* were stimulated for the indicated times with R1881 alone or together with PTHrP. Lysates were analyzed by immunoblotting with anti-AR and anti-tubulin antibodies. Densitometric quantitation was performed for three independent experiments. *Points,* mean fold change; *bars,* SE. *, $P < 0.05$. *D,* RNA extracted from LNCaP cells treated for 6 h with PTHrP and R1881, alone or in combination, was analyzed by quantitative real-time PCR using primers specific to the indicated androgen-regulated genes. Experiments were repeated thrice. The results were normalized to β-glucuronidase reference gene and are expressed as fold change from vehicle treatment. *, $P < 0.05$.

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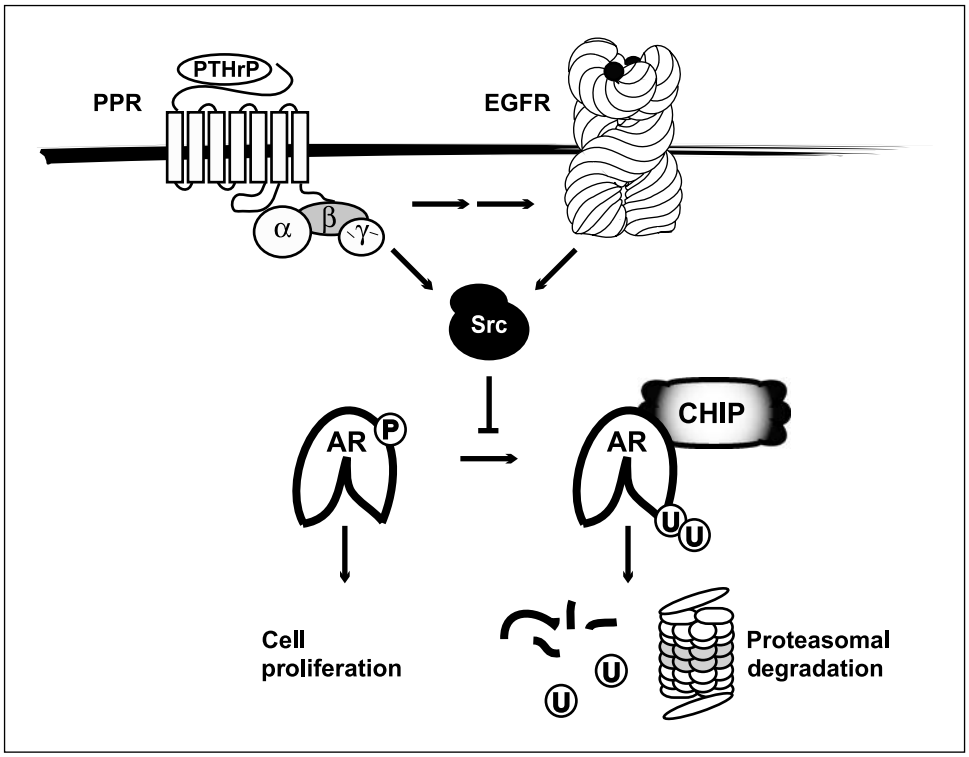

Figure 6. Model delineating the signaling pathways mediating PTHrP-enhanced AR protein stabilization. Binding of PTHrP to its cognate receptor results in the transactivation of EGFR and Src. Tyrosine phosphorylation of AR catalyzed by Src results in the sequestration of AR from chaperone and CHIP complexes that mediate its proteasomal degradation. Increased AR protein levels enhance the ability of prostate cancer cells to proliferate.

tissues and regression of prostate cancer (40). In comparing the growth properties of LNCaP cells, an androgen-dependent human prostate cancer cell line, in the presence of PTHrP, a disparity was observed in the levels of androgen required for cell proliferation. LNCaP cells treated with PTHrP required androgen concentrations 2 orders of magnitude lower for growth stimulation (Fig. 1), suggesting that in the setting of low levels of androgen following pharmacologic or surgical castration PTHrP may enhance the proliferation of prostate cancer cells. The proliferative advantage provided by PTHrP seems to be associated with a complex pattern of androgen-regulated genes, as indicated by the small sampling of AR targets we examined. Further analysis will be required to determine how the global pattern of gene expression contributes to enhanced prostate cancer cell proliferation in the presence of PTHrP and low androgen (Fig. 5*D*).

Under low androgen conditions, augmentation of LNCaP proliferation or AR protein concentration in response to PTHrP is mediated through EGFR and Src kinase activation (Figs. 1 and 3). Transactivation of this pathway in response to neuropeptide stimulation has been shown by Gschwind and colleagues (41) and is consistent with our work in PC3 cells (25). In the current study, phosphorylation of EGFR was detected after 1 hour of PTHrP treatment corresponding with increased AR protein levels. Activation of Src seems to be an important step in the regulation of AR protein levels in the presence of PTHrP, as its inhibition or siRNA knockdown abrogates PTHrP-induced AR accumulation (Fig. 3). Src association with AR occurs either directly or in a ternary complex with the scaffold protein RACK1 (19, 42), resulting in AR phosphorylation, increased AR activity, and enhanced LNCaP cell proliferation (21). Our data agree with these findings showing that AR tyrosine phosphorylation is increased in response to PTHrP (Fig. 4) and that PTHrP-mediated cell proliferation requires AR as well as Src activation (Fig. 1). Src can also interact directly with Hsp90 chaperone complexes, and its ability to modulate client protein turnover further supports its involvement in the modulation of AR protein levels (43). Chaperone proteins maintain AR in a partially unfolded ligand-free state to prevent aggregation of the protein but may also modulate AR protein degradation, nuclear translocation, and transactivation (32). It is possible that Src could modify or recruit various components of the complex to influence multiple aspects of AR downstream signaling. Our work favors the hypothesis that phosphorylation of AR modulates its ability to associate with the chaperone complex. We observed that interaction of AR with Hsp90 as well as Hsp70 is decreased in the presence of PTHrP (Fig. 4; data not shown).

Molecular chaperones regulate the proteasome-dependent degradation of several classes of proteins, including steroid receptors, membrane proteins, and kinases. One link between chaperones and the proteasome is CHIP. CHIP contains tetratricopeptide repeats for interaction with Hsp70 and Hsp90 and a modified form of the Ring finger domain found in some ubiquitin ligases (44). In conjunction with the UBCh5 group of E2 enzymes, CHIP promotes ubiquitination of glucocorticoid receptor, CFTR, and Raf kinase (45–47). Overexpression studies have revealed that CHIP prevents the accumulation of AR and stimulates its ubiquitination in LNCaP or HeLa cells (34, 48). Our observations in LNCaP cells with endogenous CHIP levels are consistent with these studies and show that AR polyubiquitination is reduced in response to PTHrP stimulation and correlates with reduced CHIP binding.

The interaction of CHIP with AR has been characterized *in vitro* and was shown to depend on both unphosphorylated and serine/threonine phosphorylated peptides of AR (49). In this study, we find that the interaction of CHIP with the phosphorylation-deficient AR-Y534F is enhanced, suggesting that, unlike serine/

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threonine phosphorylation, tyrosine phosphorylation may disrupt the AR/CHIP complex. Serine phosphorylation of AR has also been described to enhance its proteasomal degradation. Serine phosphorylation by Akt is postulated to increase AR ubiquitination by Mdm2 and lead to its degradation (49). Further analysis will be required to elucidate the relationship between these apparently antagonistic phosphorylation events in the regulation of AR/CHIP interaction and their role in the regulation of AR protein turnover.

In summary, PTHrP enhances the proliferation of LNCaP cells at low concentrations of R1881 by modulating androgen-induced stabilization of AR. Under low androgen, PTHrP functions as a compensatory signal to modulate the AR protein life cycle through pathways that involve the activation of EGFR and Src. We show that PTHrP-induced transactivation of EGFR, Src, and consequent AR tyrosine phosphorylation sequesters a subpopulation of AR protein from chaperone/CHIP complexes that normally regulate the proteasomal-dependent degradation of the protein (Fig. 6). This study provides one mechanistic explanation whereby increased neuroendocrine differentiation promotes the progression of prostate cancer through the production of factors that stabilize AR protein. The findings reveal potential new sites of therapeutic intervention that can be combined with androgen ablation.

Disclosure of Potential Conflicts of Interest

S.J. Parsons: consultant/advisory board, Bristol Myers Squibb. The other authors disclosed no potential conflicts of interest.

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Correction: Article on PTHrP Stabilizes AR and Enhances Prostate Cancer Growth

In the article on how PTHrP stabilizes AR and enhances prostate cancer growth in the September 15, 2009 issue of *Cancer Research* (1), the graph legend in Fig. 5C should indicate AR-Y534F, not AR-Y534C, for gray lines.

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The Neuroendocrine-Derived Peptide Parathyroid Hormone–Related Protein Promotes Prostate Cancer Cell Growth by Stabilizing the Androgen Receptor

John DaSilva, Daniel Gioeli, Michael J. Weber, et al.

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