Receptor for Activated C Kinase 1 (RACK1) and Src Regulate the Tyrosine Phosphorylation and Function of the Androgen Receptor

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Abstract

The androgen receptor (AR) remains functionally important in the development and progression of prostate cancer even when the disease seems androgen "independent." Because signal transduction by growth factor receptors increases in advanced prostate cancer and is capable of sensitizing the AR to androgen, there is considerable interest in determining the mechanisms by which signaling systems can modulate AR function. We show herein that the adaptor/scaffolding protein receptor for activated C kinase 1 (RACK1), which was previously reported to interact with the AR, modulates the tyrosine phosphorylation of AR and its interaction with the Src tyrosine kinase. We also show that down-regulation of RACK1 by short interfering RNA inhibits growth and stimulates prostate-specific antigen transcription in androgen-treated prostate cancer cells. Our results suggest that RACK1 mediates the cross-talk of AR with additional binding partners, such as Src, and facilitates the tyrosine phosphorylation and transcriptional activity of the AR. (Cancer Res 2006; 66(22): 11047-54)

Introduction

Prostate cancer initially presents as an androgen-dependent disease, and thus, hormone ablation is often used as a frontline therapy. However, the disease usually progresses to a state that is functionally independent of androgen. In most cases of advanced prostate cancer, the androgen receptor (AR) continues to be required and functions in the continued expression of "androgen-dependent" genes. In these cases, the AR becomes hypersensitive to androgen and is able to function even at castrate levels of hormone.

Substantial evidence indicates that the activity of the AR can be regulated not only by androgen but also by cross-talk with protein kinase signal transduction pathways. Several of these pathways have been shown to increase in activity during prostate cancer progression, and it is likely that this cross-talk plays a significant role in progression to hormone independence (1–3). However, the mechanisms by which these signaling pathways affect AR function are not clear. The kinase pathways may modulate AR via phosphorylation of either AR itself or its coregulators. Analyzing the mechanisms that integrate steroid receptor function with regulatory protein kinase cascades is likely to be important for understanding progression to androgen independence and identifying ways to prevent or reverse that process.

Scaffold proteins represent one mechanism for integrating diverse signaling pathways. These proteins cluster signaling molecules to facilitate tight regulation of cellular pathways as well as controlled cross-talk with different signaling cascades. One such scaffold is receptor for activated C kinase 1 (RACK1), a 36-kDa cytosolic protein containing seven internal Trp-Asp 40 (WD40) repeats homologous to the G protein β subunit, and the recently described mitogen-activated protein kinase (MAPK) scaffold MAPK organizer 1 (MORG1; ref. 4). RACK1 was initially identified as an anchoring protein for protein kinase C (PKC; ref. 5), with PKCβII seemingly being the preferred binding partner (6). Later reports showed that RACK1 associates with a large number of signaling proteins, including Src family kinases, integrin β subunit, PDE4D5, signal transducers and activators of transcription 1 (STAT1), insulin-like growth factor-I receptor, and others (7–11). RACK1 is ubiquitously expressed in a wide range of tissues, including brain, liver, and spleen, suggesting that it has an important functional role in many cell types (12). Interestingly, RACK1 has been recently identified as an AR-interacting protein, which facilitates ligand-independent AR nuclear translocation on activation of PKC (13). These findings raise the possibility that RACK1 may function as a scaffolding protein that links the AR to inputs from several signaling pathways.

Here, we confirm the AR-RACK1 binding interaction and show that it is regulated by androgen. We show that another RACK1 partner, the tyrosine kinase Src, also binds to the AR and that this binding requires Src kinase activity. We also show that androgen rapidly stimulates Src activation and AR tyrosine phosphorylation, which is dependent on RACK1. Collectively, the data point to the existence of an androgen-regulated ternary interaction between RACK1, active Src, and the AR, leading to AR tyrosine phosphorylation. To determine the functional significance of the RACK1-AR interaction, we examined the effects of RACK1 knockdown on growth and prostate-specific antigen (PSA) gene expression in LNCaP cells. We found that RACK1 knockdown inhibits cell proliferation induced by androgen, whereas it up-regulates PSA transcription in hormone-treated cells.

The present observations support a role for RACK1 in coordinating the integration of androgen and tyrosine kinase signaling in the function of the AR. We hypothesize that these interactions play a role in the progression of prostate cancer to androgen independence.

Materials and Methods

Cell culture, transfections, and reagents. LNCaP cells (a gift from Dr. L. Chung, Emory University, Atlanta, GA) were grown in T-medium

supplemented with 5% (v/v) fetal bovine serum (FBS; Hyclone, South Logan, UT). COS1 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS. All media were from Life Technologies (Grand Island, NY). Transfections were done using LipofectAMINE (LNCaP) or LipofectAMINE 2000 (COS1; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The following constructs (5.0 μg of DNA/100-mm dish) were used for transient expression: wild-type (wt) tagged AR and RACK1, wtSrc, activated Src [pp60(527F)], COOH-terminal Src kinase (CSK), and catalytically inactive Src mutant (K-Src). FLAG-mutated (mt) AR (Y534C) was constructed using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Commercially available reagents and antibodies included the following: methyltrienolone (R1881, Perkin-Elmer, Boston, MA); dihydrotestosterone (DHT); epidermal growth factor (EGF); anti-FLAG M2 (Sigma, St. Louis, MO); PP2 and SU6656 (Calbiochem, San Diego, CA); anti-AR (PG-21, Upstate Biotechnology, Lake Placid, NY); anti-RACK1 (BD Biosciences, San Jose, CA or Santa Cruz Biotechnology, Santa Cruz, CA); anti-CSK and anti-phosphotyrosine (PY-99, Santa Cruz Biotechnology); and anti-HA (12CA5), anti-phosphorylated Src (Tyr[416]), and anti-Src (Cell Signaling Technology, Beverly, MA). Chemicals and reagents were from Sigma unless otherwise specified.

Immunoprecipitation. Cells were lysed 24 hours after transfection, and the lysates were subjected to immunoprecipitation as detailed in ref. 4. For the detection of AR phosphorylation, cells were lysed in radio-immunoprecipitation assay (RIPA) buffer [137 mmol/L NaCl, 20 mmol/L Tris (pH 7.4), 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 2 mmol/L EDTA supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, and protease inhibitors] and immunoprecipitation was carried out as described (14). The immunocomplexes were then subjected to SDS-PAGE and Western blotting using monoclonal anti-phosphotyrosine antibody. Immunoreactions were detected by using enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ).

RNA interference. Three different 21-base double-stranded small interfering RNAs (siRNA) against RACK1 were synthesized and purified by Ambion, Inc. (Austin, TX). The target sequences were 5′-GGGAUGAG-ACCAACUAUGGtt (KD1), 5′-GGUAUGGAACCUGGCUAACtt (KD2), and 5′-GGGAAAGAUCAUUGUAGAUtt (KD3) and were controlled by BLAST search to verify sequence specificity. Transfection of RACK1 siRNA was done in LNCaP cells by using Oligofectamine (Invitrogen) in Opti-MEM I medium without serum. For Src silencing, we used a Validated Stealth RNAi DuoPak (Invitrogen) according to the manufacturer's protocol. After transfection, the cells were grown in phenol red–free medium containing charcoal stripped serum. A nonspecific control duplex-XIII (Dharmacon Research, Lafayette, CO) was used as a siRNA silencing control. Maximal knockdown of RACK1 and Src was observed on day 3 after transfection; knockdowns were assessed by Western blotting.

In vitro **kinase assay.** COS1 cells were transfected with either FLAG-wtAR or FLAG-mtAR (Y534C). Lysates were immunoprecipitated using M2 anti-FLAG affinity resin. The FLAG fusion proteins were then eluted under acidic conditions with 0.1 mol/L glycine (pH 3.5) for 30 minutes at 4°C, neutralized with 0.5 mol/L HEPES (pH 7.4), and then subjected to *in vitro* kinase assay using NH₂-terminal 6His-tagged recombinant, full-length, human Src (Upstate Biotechnology) in kinase reaction buffer [25 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 2 mmol/L $MgCl_2$] containing 1 mmol/L ATP. Src activity was controlled using acid-denatured enolase as substrate (2 mg/mL; Sigma) in the presence of 10 μCi [γ-^{32}P]ATP (Perkin-Elmer). The kinase reaction mixtures were incubated for 30 minutes at 30°C and terminated by the addition of sample buffer and boiling for 5 minutes. The samples were then subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody or autoradiography accordingly.

Real-time PCR assay. Total RNA was extracted from cells using the RNeasy kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 and 280 nm. An additional on-column DNase I digestion step was done, and RNA samples were quantified again by using RiboGreen (Invitrogen). Total RNA (1 μg) from each sample was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Synthesized cDNA was then combined in a PCR using PSA primers, 5′-TGGTGCATTACCGGAAAGTGGATCA-3′ (forward) and 5′-GCTTGAGT-CTTGGCCTGGTCATTTC-3′ (reverse), and β-glucuronidase (GUS), 5′-CCG-ACTTCTCTGACAACCGACG-3′ (forward) and 5′-AGCCGACAAAATGCC-GCAGACG-3′ (reverse), as the housekeeping gene. Quantitative real-time PCR was done on a LightCycler by using SYBR Green PCR Master Mix (Bio-Rad). For each amplification run, PSA and GUS standard curves were independently generated by assaying the same cDNA samples in duplicates.

Growth assay. Cell growth was determined by [³H]thymidine incorporation. LNCaP cells were seeded in duplicates 2 days before transfection with the various RACK1 siRNAs (KD1-KD3). Three days after transfection, the cells were stimulated and then labeled with 1 μCi/well of [³H]thymidine (Perkin-Elmer) and incubated for 6 hours in a 37°C humidified 5% CO_2 incubator. The labeled cells were washed twice with PBS, precipitated with
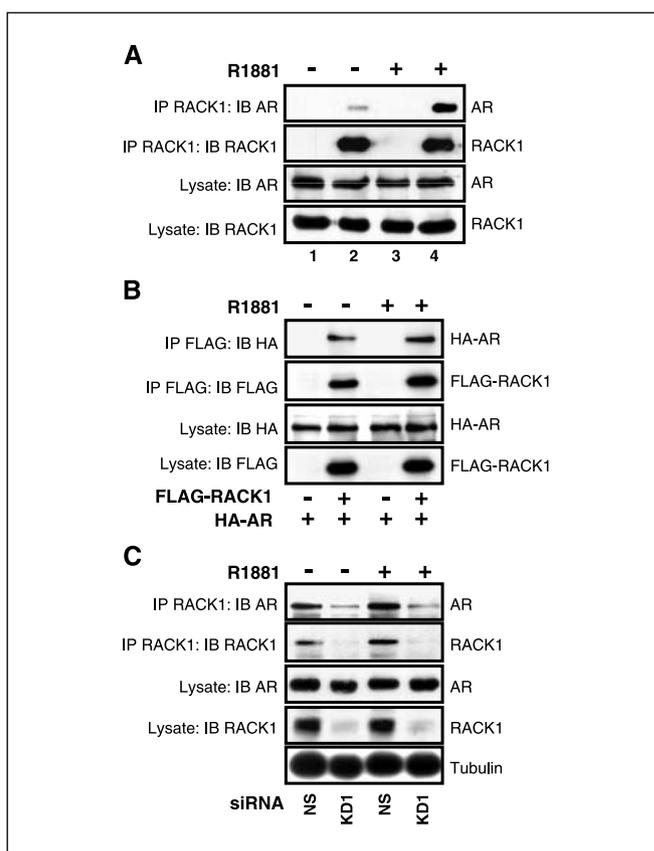
Figure 1. Specificity of *in vivo* and *in vitro* RACK1-AR interaction. *A,* protein extracts from LNCaP cells were used to determine endogenous RACK1-AR interaction. Anti-RACK1 antibody coupled to protein G agarose was used to immunoprecipitate RACK1 and associating AR. *Lanes 1* and *3,* cell extracts immunoprecipitated with a nonspecific mouse IgG antibody; *lanes 2* and *4,* cell extracts immunoprecipitated with anti-RACK1. Results are representative of three independent experiments. *B,* COS1 cells were cotransfected with FLAG-RACK1 and HA-AR as indicated and serum starved (16 hours) in phenol red–free DMEM before stimulation. The cells were then treated with R1881 (1 nmol/L) or ethanol (vehicle) for 1 hour. Cell extracts were immunoprecipitated using M2 anti-FLAG affinity resin. Immunoprecipitates were probed with either anti-FLAG or anti-HA antibodies. Results are representative of three independent experiments. *C,* LNCaP cells were transfected with either a nonspecific siRNA (*NS*) or a RACK1 siRNA (*KD1*) as indicated. At day 3 after transfection, the cells were serum starved for 4 hours in phenol red–free RPMI 1640 and then stimulated with either R1881 (1 nmol/L, 1 hour) or left untreated. Cell extracts were subjected to immunoprecipitation, and the immunoprecipitates were probed with either anti-AR, anti-RACK1, or anti-Src antibodies.

Figure 2. Src interacts with AR in prostate cancer cells. *A,* serum-starved (16 hours) LNCaP cells were either pretreated with PP2 (20 μmol/L, 30 minutes) or SU6656 (5 μmol/L, 1 hour) or left untreated. The cells were then stimulated with R1881 (1 nmol/L) for the indicated times and subjected to immunoprecipitation using anti-Src. The immunoprecipitates were probed with either anti-phosphorylated Src (*pSrc*; Y416) or anti-Src antibodies. Results are representative of three independent experiments. *B,* LNCaP cells were cotransfected with FLAG-AR, HA-RACK1, and wtSrc as indicated. Twenty-four hours after transfection, the cells were serum starved (4 hours) in phenol red–free RPMI 1640 before stimulation and then treated with R1881 (1 nmol/L) or ethanol (*EtOH*) for 30 minutes. Cell extracts were immunoprecipitated using M2 anti-FLAG affinity resin. Immunoprecipitates were probed with anti-FLAG, anti-phosphorylated Src (Y416), anti-Src, or anti-HA antibodies. *C,* serum-starved (16 hours) LNCaP cells were either pretreated with PP2 (20 μmol/L, 30 minutes) or left untreated. The cells were then stimulated with R1881 (1 nmol/L) and subjected to immunoprecipitation using either anti-Src or anti-RACK1 coupled to protein G agarose. The immunoprecipitates were subjected to SDS-PAGE and Western blotting. *D,* LNCaP cells were transfected with either a nonspecific siRNA (*NS*) or RACK1 siRNA (*KD*) as indicated. At day 3 after transfection, the cells were serum starved for 4 hours in phenol red–free RPMI 1640 and then stimulated with either R1881 (1 nmol/L, 1 hour) or left untreated. Cell extracts were subjected to immunoprecipitation using anti-Src coupled to protein G agarose, and the immunoprecipitates were probed with either anti-AR, anti-Src, or anti-RACK1 antibodies.
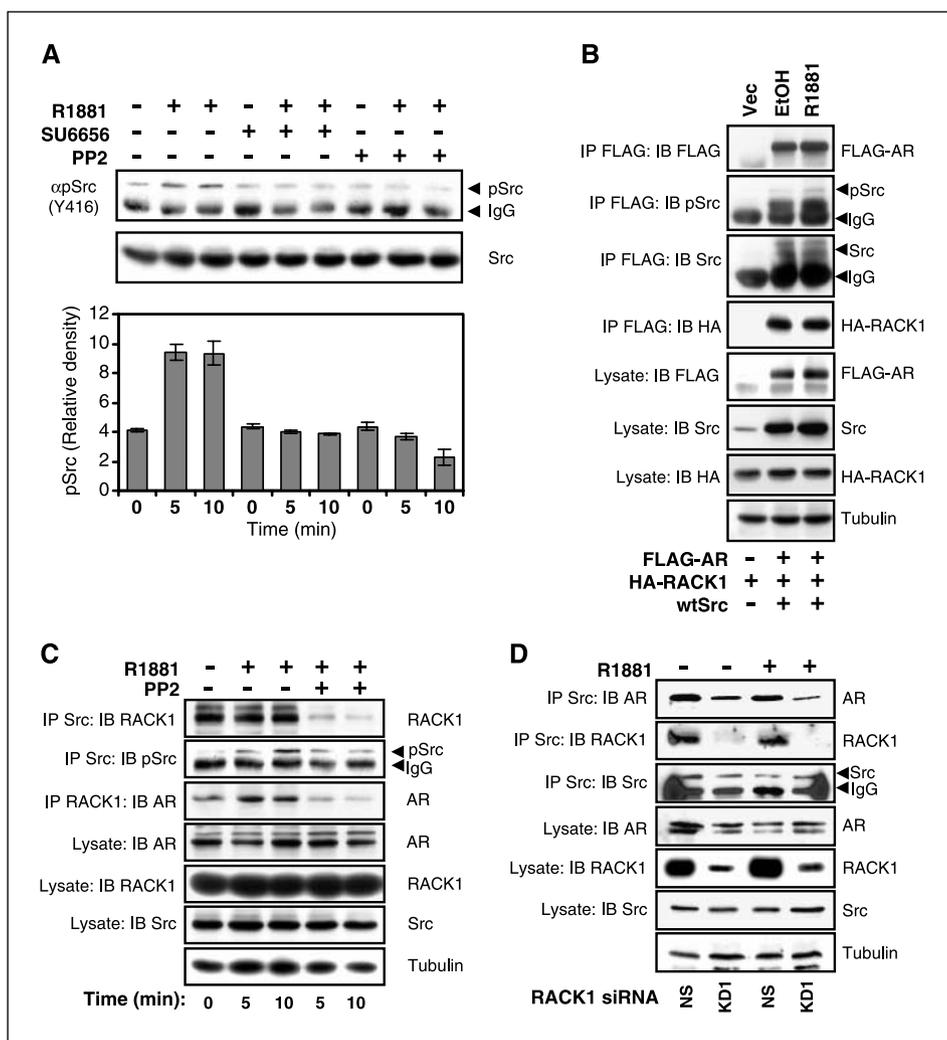
10% trichloroacetic acid, and lysed with 0.2 mol/L NaOH containing salmon sperm DNA (40 μg/mL). The radioactivity was counted by liquid scintillation spectrometry in a scintillation spectrometer.

Results

Regulation and specificity of RACK1-AR interaction. A previous study showed that RACK1 is an AR-interacting protein as shown by yeast two-hybrid screening and minimal domain mapping (13). We further examined the regulation of RACK1-AR interactions in living cells and tested the specificity of the interaction by knockdown with siRNA (Fig. 1). LNCaP prostate cancer cells, which express the AR, were serum starved in medium without phenol red for 16 hours and then treated with either the synthetic androgen R1881 (1 nmol/L) or ethanol (vehicle) for 1 hour (Fig. 1A). Androgen treatment of LNCaP cells significantly increased the level of endogenous RACK1-associated AR compared with untreated cells, although the level of immunoprecipitated RACK1 remained unchanged. In contrast, androgen treatment of COS1 cells transiently expressing AR by exogenous expression did not significantly affect the RACK1-AR interaction and the levels of immunoprecipitated proteins (Fig. 1B), presumably as a consequence of high levels of AR expression. The specificity of the intracellular RACK1-AR interaction was examined by RACK1

knockdown using siRNA. As shown in Fig. 1C, RACK1 silencing by KD1 siRNA decreased the amount of coimmunoprecipitated AR. We examined two additional RACK1 siRNAs (KD2 and KD3) and found that they had a similar, although less efficient, effect (Fig. 5C; data not shown).

AR interacts with Src in prostate cancer cells. RACK1 has previously been shown to directly bind and inhibit Src family kinases in NIH3T3 cells (7). We therefore examined possible interactions between AR, RACK1, and Src. We first asked whether Src is activated in LNCaP cells in response to androgen treatment. Phosphorylation of Src on Tyr[416] was detected on treatment with R1881 shortly after stimulation. Pretreatment of cells with the Src inhibitors PP2 (20 μmol/L, 30 minutes) or SU6656 (5 μmol/L, 1 hour) inhibited the androgen-induced Src activation (Fig. 2A) as expected.

To determine whether Src interacts with AR, we transfected LNCaP cells with vectors encoding FLAG-AR, HA-RACK1, and wtSrc. The cells were then stimulated with either R1881 or ethanol for 10 minutes, and the lysates were subjected to immunoprecipitation. As shown in Fig. 2B, both Src and RACK1 coimmunoprecipitated with FLAG-AR. Furthermore, R1881 treatment increased the amount of active pY416 Src pulled down, which likely reflects an increase in Src activity on hormone treatment.

We next examined the involvement of Src in RACK1-AR interaction and found that binding of RACK1 both to AR and to Src is abolished when Src activity is inhibited by PP2 in androgen-treated cells (Fig. 2C). Our results agree with previous findings showing that Src activity is required for RACK1 binding to Src and tyrosine phosphorylation of RACK1 (15).

The specificity of the endogenous RACK1-AR-Src interactions was examined by siRNA knockdown of RACK1 in LNCaP cells followed by immunoprecipitation with anti-Src antibody. As shown in Fig. 2D, RACK1 silencing by siRNA decreased the amount of coimmunoprecipitated AR. Collectively, these findings suggest that the AR interacts with Src and RACK1 in cells to form oligomeric protein complexes and that these complexes depend on Src activity.

Tyrosine phosphorylation of AR and its regulation by RACK1. Phosphorylation of steroid hormone receptors plays a role in various processes and often facilitates the recruitment of coactivators, cooperates with the ligand to enhance transcriptional activation, or contributes to the termination of the ligand response by inducing DNA dissociation or receptor degradation or through decreasing ligand affinity (16–18). By using a combination of peptide mapping, Edman degradation, and tandem mass spectrometry, seven major phosphorylation sites on the AR have been previously identified in our laboratory (2). Although, two-dimensional phosphopeptide mapping studies have shown that only serine residues in the AR are phosphorylated (2, 19), the NetPhos approach predicted several tyrosine residues as phosphorylation consensus sites for AR (20). Thus, additional potential phosphosites, including tyrosine residues, may exist and are yet to be identified.

We examined whether AR can be tyrosine phosphorylated and if so whether RACK1 or Src binding plays a role in the process. As shown in Fig. 3A, treatment of LNCaP cells with androgen (1 nmol/L) rapidly and transiently induces tyrosine phosphorylation of AR. The transient nature of this phosphorylation accounts for the failure to detect it in previous studies (2, 19). We also found that EGF stimulation induces tyrosine phosphorylation of AR, suggesting that this modification represents an integration of signaling to the AR from different agonists. Our results are further supported by a recent study (21) showing that AR tyrosine phosphorylation on
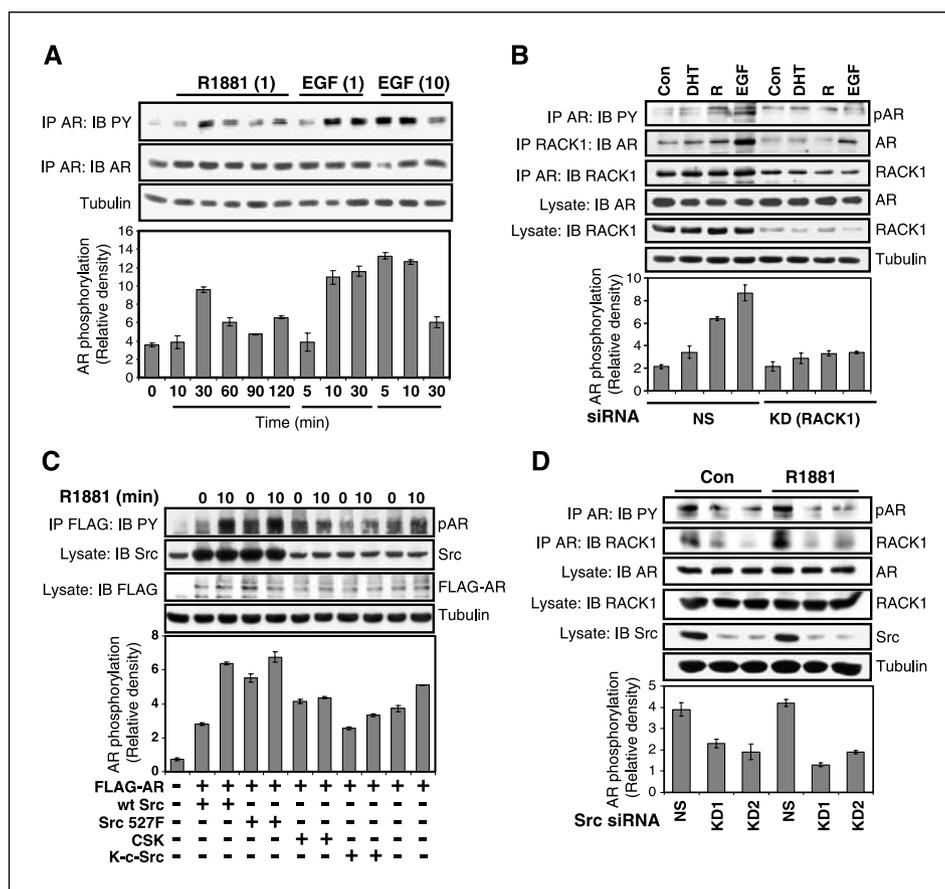
Figure 3. AR is tyrosine phosphorylated in LNCaP cells. *A,* LNCaP cells were serum starved (16 hours) in phenol red–free RPMI 1640 and then stimulated with either R1881 (1 nmol/L) or EGF (1 and 10 ng/mL) for the indicated times. Cell extracts prepared in RIPA buffer were subjected to immunoprecipitation as indicated and probed with anti-phosphotyrosine (*PY*) antibody. Results are representative of three independent experiments. *B,* LNCaP cells were transfected with either a nonspecific siRNA (*NS*) or a RACK1 KD1 siRNA (*KD*). At day 3 after transfection, the cells were serum starved for 4 hours and then treated with DHT (1 nmol/L), R1881 (1 nmol/L), and EGF (10 ng/mL) for 10 minutes or left untreated (*Con*). Cell extracts were subjected to immunoprecipitation as indicated. Results are representative of three independent experiments. *C,* LNCaP cells were cotransfected with either FLAG-AR alone or FLAG-AR with wtSrc, Src527F, CSK, or K-Src. One plate was transfected with vector alone as control. Twenty-four hours after transfection, the cells were serum starved (4 hours) in phenol red–free RPMI 1640 before stimulation and then treated with R1881 (1 nmol/L) or ethanol (vehicle) for 10 minutes. Cell extracts were immunoprecipitated using M2 anti-FLAG affinity resin. Immunoprecipitates were probed with anti-phosphotyrosine antibody. Results are representative of three independent experiments. *D,* LNCaP cells were transfected with either a nonspecific siRNA (*NS*) or two Src siRNAs (*KD1* and *KD2*) targeting different regions of the gene. At day 3 after transfection, the cells were serum starved for 4 hours and then treated with R1881 (1 nmol/L) or ethanol (*Con*) for 10 minutes. Cell extracts were subjected to immunoprecipitation as indicated.

Figure 4. Src phosphorylates AR. *A,* COS1 cells were transfected with either FLAG-wtAR or FLAG-mtAR (Y534C). Lysates were immunoprecipitated using M2 anti-FLAG affinity resin. The FLAG fusion proteins were eluted with 0.1 mol/L glycine (30 minutes, 4°C) and subjected to *in vitro* kinase assay using active recombinant Src (30 minutes, 30°C) as described in Materials and Methods. The samples were then subjected to SDS-PAGE and Western blotting using anti-phosphotyrosine antibody. *B,* activity of Src toward acid-denatured enolase (2 mg/mL) was determined by *in vitro* kinase assay in the presence of 10 μCi [γ-^{32}P]ATP. The phosphorylation was detected by SDS-PAGE followed by autoradiography.

Y534 is induced in prostate cancer cells in response to other growth factors, including heregulin and IL-6, and elevated in hormone-refractory prostate tumor xenografts and patient samples. Taken together, these observations show that tyrosine phosphorylation of AR occurs in response to diverse agonists in addition to androgen.

To assess whether RACK1-AR interaction is involved in the tyrosine phosphorylation of AR, we examined the effect of siRNA-induced RACK1 silencing. As shown in Fig. 3*B*, RACK1 down-regulation by siRNA significantly reduced AR tyrosine phosphorylation in response to DHT, R1881, or EGF, suggesting a role for RACK1 in this AR modification.

To examine the role of Src kinase activity in the tyrosine phosphorylation of the AR, LNCaP cells were transiently transfected with either FLAG-AR alone or FLAG-AR with wtSrc, an activated Src mutant (Src527F), CSK, or kinase-dead K-Src. Overexpression of wtSrc or Src527F significantly increased AR tyrosine phosphorylation compared with cells transfected with FLAG-AR alone (Fig. 3*C*). On the other hand, AR tyrosine phosphorylation was reduced by either overexpression of CSK, which serves as a Src dominant interfering kinase, or by the K-Src mutant, which acts as a dominant-negative competitor. The involvement of Src in AR phosphorylation was further supported by showing that Src knockdown by siRNA significantly reduced the tyrosine phosphorylation of AR and AR binding to RACK1 (Fig. 3*D*). Taken together, these results strengthen the suggestion that Src is involved in the RACK1-mediated tyrosine phosphorylation of AR.

AR is phosphorylated by Src *in vitro*. To further confirm that AR is specifically phosphorylated by Src, the ability of Src to phosphorylate AR was determined by *in vitro* kinase assays using immunoprecipitated FLAG-wtAR and a FLAG-mtAR (Y534C) as substrates. As shown in Fig. 4*A*, wtAR was extensively phosphorylated by a purified active Src recombinant kinase as detected by anti-phosphotyrosine immunoblotting. Conversely, mutation of Tyr^{534} on AR prevented its phosphorylation by Src. Acid-denatured enolase was used as a positive control for Src activity (Fig. 4*B*).

RACK1 regulates AR function. To assess the functional significance of the RACK1-AR interaction, the role of RACK1 in cell growth and AR transactivation was examined in androgen-treated LNCaP cells transfected with three different RACK1 siRNAs (KD1-KD3; Fig. 5). Proliferation of LNCaP cells treated with a low concentration of R1881 (0.1 nmol/L, 48 hours) was significantly inhibited by all three RACK1 siRNAs (Fig. 5*A*). On the other hand, RACK1 knockdown potentiated the induction of PSA transcription by R1881 (0.1 nmol/L, 12 hours) in these cells (Fig. 5*B*). It should be noted that, although the various RACK1 siRNAs had similar effects on the androgen-induced PSA gene expression, KD2 and KD3 were quantitatively less effective both in knockdown (Fig. 5*C*) and in transcriptional effects.

Discussion

To understand the mechanisms by which growth factor signaling can modulate the activity of the AR, we have examined the physical and functional interactions between the AR, the

Figure 5. RACK1 regulates AR function. *A,* cells were transfected with a nonspecific siRNA (*NS*) or three different RACK1 siRNAs (*KD1, KD2,* and *KD3*) targeting the *RACK1* gene as indicated. At day 3 after transfection, the cells were treated with 0.1 nmol/L R1881 (+) or vehicle (−) for 48 hours. Following stimulation, [³H]thymidine (1 μCi/well) was added for 6 hours and thymidine incorporation was measured as described in Materials and Methods. *B,* LNCaP cells were transfected as above, and at day 3 after transfection, the cells were treated with either 0.1 nmol/L R1881 (+) or vehicle (−) for 12 hours. Total RNA prepared from LNCaP cells was used as a template for real-time PCR as described in Materials and Methods. *C,* effectiveness of RACK1 knockdown in LNCaP cells measured by protein expression.

scaffold protein RACK1, and the signaling kinase Src. Our findings provide evidence that RACK1 mediates androgen and growth factor cross-talk by facilitating the interaction of the AR with the Src tyrosine kinase.

RACK1, which was initially identified as a binding protein for PKC (5), was subsequently shown to interact with a wide range of signaling molecules and thus can serve as a platform for integrating diverse signaling activities. Src and the AR are among the reported binding partners for RACK1 (7, 13). In our study, we showed that the RACK1-AR interaction occurs in LNCaP cells at endogenous levels of protein expression and that the interaction is enhanced when cells are treated with androgen. Interestingly, we found that, when RACK1 and the AR are overexpressed by transient transfection, the dimeric interaction became constitutive even in the absence of androgen. We suspect that the high levels of RACK1 and AR expression that occur when the proteins are transiently overexpressed saturate the interaction so that an affinity effect due to R1881 treatment is obscured.

We also found that the Src protein coimmunoprecipitated with the AR as described previously (22) and that the AR became transiently tyrosine phosphorylated in response to androgen or EGF. Both AR-Src binding and androgen-induced AR phosphorylation were inhibited by blocking Src activity or by knocking down RACK1 with siRNA. These data suggest that activation of Src results in binding of RACK1 to Src and this complex recruits AR, which then becomes phosphorylated on tyrosine. Because we showed that Src can phosphorylate the AR directly *in vitro*, it is attractive to hypothesize that Src is the AR protein tyrosine kinase *in vivo*, but this remains to be proven.

Knockdown of RACK1 with siRNA disrupted the AR-Src interaction (Fig. 2D), and knockdown of Src disrupted the RACK1-AR interaction (Fig. 3D). In addition, RACK1 knockdown inhibited AR tyrosine phosphorylation (Fig. 3B), although it has been shown that RACK1 silencing does not inhibit Src activity (23). These data suggest that RACK1, Src, and the AR form a ternary complex (although a larger complex or a dynamic series of dimeric complexes is possible). Because Src itself can be activated by numerous extracellular signals, the RACK1-Src-AR complex could potentially allow the AR to monitor a wide range of regulatory activities in addition to androgen levels.

Considerable published information exists about the molecular requirements for interaction between RACK1 and its binding partners. The interaction of Src and RACK1 is partly mediated by the SH2 domain of Src and by the sixth WD repeat of RACK1, which contains the Src phosphorylation site (Tyr^{246}). Phosphorylation of this site is apparently involved in the RACK1-Src interaction as a RACK1 mutant, Y246F, is unable to bind Src (8). In addition, the interaction of Src and RACK1 is enhanced by serum or platelet-derived growth factor stimulation, by PKC activation, and by the Src-induced tyrosine phosphorylation of RACK1 itself (8). The sixth WD repeat of RACK1 also contains the binding sites for PKC, β-integrin, and other proteins, consistent with the role of RACK1 as an integrator of diverse signaling pathways (12).

The interaction of Src with the AR likely occurs via interactions between the SH3 domain of Src and proline-rich sequences in the AR (23, 24). The progesterone receptor (PR) also binds Src (24) via interactions between a polyproline motif in the PR and the SH3 domain of Src (25). Moreover, Src has previously been found to bind to and phosphorylate the estrogen receptor (ER) and regulate

the hormone binding capacity of ER and its dimerization (26), and the association of Src with ER and AR in response to steroid is apparently involved in triggering of prostate cancer cell proliferation (22).

The concept that the interaction of steroid receptors with kinases is mediated by scaffold proteins is supported by additional reports showing that the scaffolding protein modulator of nongenomic actions of the ER (MNAR) physically bridges ER and Src in a trimeric complex, resulting in activation of Src and enhanced ER transcription (27). MNAR has been found to interact directly with both ER and Src in the cytosol, stabilizing the interaction and leading to Src activation by unfolding Src and inducing Y416 phosphorylation (28). Interestingly, MNAR was recently shown to coimmunoprecipitate with the AR in transfected COS cells (29). Similar to RACK1, which contains multiple docking sites (e.g., WD40 repeats), MNAR has several LXXLL motifs that can interact with the AF2 domain of the ER as well as several proline-rich motifs that can interact with Src or other SH3 domain-containing proteins (30).

Qiu et al. have recently identified AR tyrosine phosphorylation sites by mass spectrometry analysis (21). Data from this analysis show that Y534 of AR is a candidate phosphorylation site for Src. We confirmed these findings and found that mutation of Y534 prevents the tyrosine phosphorylation of AR by Src *in vitro*, further supporting our results.

There are several kinases in addition to Src predicted to be involved in the phosphorylation of AR. The identified phosphosites Ser^{650} and Ser^{256} are consensus sites for casein kinases 1 and 2, whereas Ser^{81} is a candidate PKC site. *In vitro* kinase studies have shown that AKT (31) and MAPK (32) are capable of phosphorylating the AR. However, other studies failed to detect AR phosphorylation changes in cells due to activation of MAPK or AKT (2). Recently, we found that activation of stress kinase signaling can regulate Ser^{650} phosphorylation and AR nuclear export through phosphorylation of this site (14). Thus, the kinases and the possible signaling pathways involved in the phosphorylation of the AR are yet to be fully elucidated.

We examined the functional significance of the RACK1-AR interaction by down-regulating RACK1 using siRNAs. Our results showed that RACK1 knockdown inhibits cell growth induced by androgen, whereas it up-regulates PSA transcription in hormone-treated LNCaP cells. This is consistent with previous findings showing that RACK1 is involved in the transactivation of AR via activation of the PKC signaling pathway, which represses PSA mRNA expression by decreasing AR recruitment to the PSA promoter (13).

Several studies have focused on the role of RACK1 in the regulation of cell growth. Overexpression of RACK1 in NIH3T3 cells was found to reduce the growth rate in both anchorage-dependent and anchorage-independent conditions mainly because of a G_1 delay, which correlates with increased levels of the cyclin-dependent inhibitors $p21^{Cip1/WAF1}$ and $p27^{KIP1}$ (7, 33). Although RACK1 has effects on general growth capacity, it is noteworthy that RACK1 knockdown in LNCaP cells almost completely inhibits the ability of androgen to stimulate growth.

Additional studies have shown that Chinese hamster ovary cells overexpressing RACK1 show decreased integrin-dependent cell migration and an increased number of actin stress fibers and focal contacts (34). Overexpression of RACK1 also led to enhanced cell spreading and increased focal adhesions, which were accompanied by increased tyrosine phosphorylation of focal adhesion kinase and

paxillin (33). Conversely, depletion of RACK1 by antisense RNA prevented cell spreading, reduced the number of focal adhesions, and inhibited growth factor–stimulated cell proliferation, which indicates that RACK1 is a possible regulator of these processes (33). Noteworthy, RACK1 was found to regulate integrin-mediated adhesion and chemotactic cell migration through its interaction with Src (35). Thus, one of the functions of RACK1 may be to control the interactions of signaling pathways involved in the coordination of adhesion, movement, and/or growth. Interestingly, a recent report has shown that RACK1 is up-regulated during angiogenesis and in human carcinomas and may contribute to tumor growth and spread (36).

An intriguing question raised by our study deals with the extent of hormone dependency of the RACK1-Src-AR complex *in vivo* and the requirement for protein colocalization. RACK1 is a cytoplasmic protein, whereas Src is cytoplasmic and associates with the cell membrane on activation. On the other hand, AR is predominantly a nuclear protein (37, 38). It is thought that the unliganded AR resides mainly in the cytoplasm, complexed with heat shock proteins and other cochaperones (39, 40). On ligand binding, the AR undergoes a conformational change and translocates into the nucleus, where it binds androgen response elements and activates gene expression. In addition, the liganded AR rapidly shuttles between the cytoplasm and the nucleus by active import and export mechanisms (14, 41, 42). Previous work showed that RACK1 regulated AR translocation in response to PKC activation (13). However, it is not known whether RACK1 plays a role also in androgen-induced translocation of the AR and in shuttling.

In summary, RACK1 acts as a scaffold protein that facilitates the association of Src with AR and is necessary for AR tyrosine phosphorylation. It is probable that the RACK1-AR complex and the consequent AR phosphorylation play a role in hormone-regulated transcriptional activation and cell growth. This may involve regulation of AR localization or the interaction with transcriptional cofactors. It is also possible that the RACK1-mediated activation of the Src pathway by androgen may lead to phosphorylation of other transcription factors that are important for AR transcriptional activity.

Acknowledgments

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Receptor for Activated C Kinase 1 (RACK1) and Src Regulate the Tyrosine Phosphorylation and Function of the Androgen Receptor

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