Androgen Induction of Prostate Cancer Cell Invasion Is Mediated by Ezrin*

Yin-Choy Chuan1, See-Tong Pang1, Angel Cedazo-Minguez2, Gunnar Norstedt3, Åke Pousette1, and Amilcar Flores-Morales2

From the 1Department of Molecular Medicine and Surgery, 2Section of Experimental Geriatrics, Karolinska Institute, Neurotec, and 3Andrology Center, Department of Medicine, Karolinska Institute, 17176 Stockholm, Sweden and 4Division of Urology, Department of Surgery, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Tao-Yuan 333, Taiwan

Ezrin is a key signaling molecule that regulates cell survival, adhesion migration, and invasion. We have previously shown that ezrin is regulated by androgen in rat prostate and that its expression is increased in prostate cancer and in prostate intraepithelial neoplasia. We have used the androgen-sensitive cell line LNCaP-FGC to investigate the role of ezrin in androgen-induced cell invasion. We found that androgen treatment of LNCaP-FGC cells induces ezrin expression, an effect that is inhibited by the androgen receptor antagonist, bicalutamide. In addition, androgen treatment induces the phosphorylation of ezrin in Thr-567 and Tyr-353 in a sequential manner. This is mediated through protein kinase C α and Src tyrosine kinase, respectively. Androgen treatment induces the translocation of both protein kinase C α and ezrin to the cell membrane and their association. Inhibition of ezrin function using short interference RNA or the overexpression of T567A and Y353F-ezrin mutants significantly reduces androgen-induced Matrigel invasion but does not affect cell proliferation or cell adhesion. Matrigel invasion of the androgen-insensitive prostate cancer cell lines PC-3 and LNCaP-R is also dependent on ezrin. In summary, we have shown that androgens regulate ezrin at transcriptional and posttranscriptional levels. Hormonal regulation of ezrin phosphorylation is required for androgen-induced cell invasion.

Prostate cancer (PCa) remains a leading cause of mortality worldwide. Despite important progress in the early diagnosis of prostate neoplasias through the measurement of prostate-specific antigen levels, ~10% of newly diagnosed patients have some evidence of locally advanced PCa and 5% already have distant metastasis at the time of diagnoses (1–3). Postmortem analysis of deaths attributed to PCa reveals that most subjects have evidence of metastatic disease (4). Some treatment alternatives exist with curative potential in the case of locally advanced PCa (5–7). In contrast, patients with evidence of distant metastasis have a very poor prognosis and no curative treatment exists (8).

Androgen ablation therapy in its many modalities has been a mainstay in the treatment of prostatic adenocarcinoma. In general, androgen deprivation induces remission in 80–90% of men with advanced PCa and results in a median progression-free survival of 12–33 months. This period is usually followed by the emergence of an androgen-independent PCa resulting in a median overall survival of 23–37 months. In metastatic PCa disease, androgen ablation is also the first line of treatment. Androgen ablation in combination with external beam radiotherapy or prostatectomy delays disease progression and results in significant survival advantage when compared with radiation therapy or prostatectomy alone (5–7, 9).

It is not entirely clear how hormone refractory cancer develops (10, 11). One hypothesis is that some of the mechanisms that are normally under androgen control become constitutively active in androgen-independent cells. This is supported by findings showing that one third of hormone refractory carcinomas contain an amplification of the androgen receptor (AR) gene (12–14), whereas 10–30% acquired point mutations, some of which cause the AR to respond to other hormones (14). Another possibility is that androgen-independent mechanisms are responsible for the phenotypic features of androgen-resistant PCa cells (10). Most likely, both androgen-dependent and -independent mechanisms are involved in PCa progression.

Despite clinical evidence indicating that androgen influences the metastatic behavior of prostate epithelial cells, our knowledge regarding how these effects are exerted remains limited. In a recent study, we have identified several novel androgen-regulated genes, some of which may be of importance in the regulation of prostate cell invasiveness (15). We have shown that ezrin expression is repressed by castration and stimulated by androgen replacement in rat ventral prostate epithelial cells. Ezrin is a linker protein with capacity to bind membrane proteins and the actin cytoskeleton. This interaction provides an intracellular scaffold for the formation of specialized membrane domains that facilitate signal transduction through a number of growth factor receptor and adhesion molecules (16). Ezrin regulates cell survival, adhesion, and migration/invasion.
phomena that are important for tumor development and progression (17, 18).

An important role for ezrin in the metastatic progression of several tumors (17–20) has been demonstrated. We have recently shown that immunoreactive ezrin content measured in radical prostatectomy samples is increased both in PCas and in high grade prostate intraepithelial neoplasia, a pre-cancerous lesion (21). Its levels correlate with adverse prognostic factors such as Gleason score and invasion of seminal vesicles (22).

Here, we have used the hormone-sensitive cell line LNCaP-FGC to study the role of ezrin in androgen-induced cell invasion. We have also explored the mechanisms whereby androgen regulates ezrin function in PCa cells.

EXPERIMENTAL PROCEDURES

Materials—Synthetic androgen R1881 was obtained from Amersham Biosciences. Androgen receptor antagonist bicalutamide (Casodex) was a generous donation from AstreaZeneca (Stockholm, Sweden). LY294002 was obtained from Cell Signaling (Beverly, MA). Y27632, calphostin C, and G66976, PP2, and N-3 were obtained from Calbiochem. U0126 was obtained from Promega (Madison, WI).

Cell Culture, Hormone, and Drug Treatments—PCa cell lines LNCaP-FGC and PC3 were obtained from the American Tissue Culture Collection (Manassas, VA). LNCaP-R cells have previously been described (23). Cell cultures were maintained as previously described (24, 25). For the experiments, cells were subcultured in 60 mm culture for 24–48 h before the medium was substituted with phenol red-free medium supplemented with 5% Dextran-coated charcoal-treated fetal bovine serum (Hyclone Inc., Logan, UT) for 24 h prior to exposure to the same medium containing 0.1% ethanol (control) or 10 nM synthetic testosterone R1881. Bicalutamide (10 μM), LY294002 (10 μM), neomycin (10 μM), U0126 (10 μM), Y27632 (20 μM), G66976 (1 μM), PP2 (10 μM), and calphostin C (0.5 μM) were added to the cells for 2 h prior to hormone stimulation. The protein synthe-
sis inhibitor cycloheximide (10 μg/ml) was added 30 min prior to hormone stimulation.

Mutagenesis and Cell Transfection—An expression con-
struct with VSV-G-tagged human wild-type ezrin (WT-ezrin) and the Y353F-ezrin mutant are a kind gift from Dr. Monique Arpin (26). The mutant T567A-ezrin was generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions and using the oligonucleotides 5’-GGGACAAGTACAAGGCG-CTGCGGCAATC-3’ and 5’-GATCTGCGCAGCGCCT-
TGTAC-TTCTCCC-3’. The annealing temperature was 58 °C.

Androgen Regulation of Ezrin Function

—RNA Extraction, cDNA Synthesis, and Real-time PCR—RNA
was extracted using TRIzol reagent (Invitrogen). Total RNA (5
μg) from different samples were first treated with DNase I (Promega) and then reverse transcribed using Superscript II (Invitrogen) in a reaction volume of 20 μl. The synthesized cDNA was used to measure the ezrin expression by real-time PCR (DNA Engine Opticon 2; M) Research, Inc.). The reactions were performed in 20 μl with 1 μl of the respective cDNA sample and 0.4 μM primers. SYBR Green I dye, hot-start iTaq DNA polymerase, buffer, and dNTPs were supplied in the iQ SYBR Green supermix (Bio-Rad). For each studied gene, a relative standard curve was constructed with serial dilutions using a pool of all cDNA samples. All the measurements were performed in triplicate for each sample and normalized to the internal control gene, β-actin. Four independent experiments were performed. The primers used were β-actin, 5’-CTGGCT-GCTGACCGAGG-3’ and 5’-GAAGGTCTCAAACATGAT-CTGGGT-3; ezrin, 5’-ACGTCTGAGATCAACAAAAGC-3’ and 5’-TTCTCCTCATAGTCCCTGAG-3’. The annealing temperature was 58 °C.

Western Blotting—LNCaP-FGC cells were cultivated and treated as described above. The cells were harvested using radioimmune precipitation buffer with protease inhibitors, and protein concentration was determined with BCA protein assay reagent (Pierce). Equal amounts of protein extracts were denatured, separated on a 12% SDS-PAGE gel (Invitrogen), and blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were then blocked in a Tris-buffered saline solution with 5% bovine serum albumin or 5% nonfat dry milk and probed with primary antibody. The membranes were further probed with horseradish peroxidase-conjugated goat anti-mouse/anti-rabbit to visualize the specific band. Mouse monoclonal anti-human ezrin antibody was obtained from Neomarker, rabbit anti-human phospho-ezrin (Thr-567) and phospho-ezrin (Tyr-353) were from Cell Signaling Technologies, monoclonal PKCα antibody was from Transduction Laboratories. Mouse anti-VSV-G antibody was obtained from Roche Applied Science. Mouse monoclonal anti-active Src (Tyr-529) and Src antibodies were obtained from BIOSOURCE.

Immunoprecipitation—Immunoprecipitations of cell ex-
tracts were performed by incubating lysates with anti-VSV-G antibody. The reaction mixture was gently rocked at 4 °C overnight. Protein G-agarose (Amersham Biosciences) was used to absorb immunocomplexes that were washed extensively with ice-cold radioimmune precipitation buffer containing 0.1% Triton X-100. The pellet was resuspended in loading buffer, boiled for 5 min, and centrifuged at 14,000 rpm for 5 min. The supernatant was collected and subjected to Western blotting.

PKCα Translocation—LNCaP-FGC cells were cultivated as described above and treated with R1881 for the times indicated in Fig. 7. After washing with phosphate-buffered saline, the cells were harvested, collected by centrifugation, and homoge-
nized by sonication in buffer containing 20 mm Tris-HCl, pH 7.5, 250 mm sucrose, 1 mm EDTA, and protease inhibitors. Cytosolic and particulate fractions were separated by ultracen-
trifugation (30 min, 100,000 × g, 4 °C). Proteins from the particulate fraction were extracted by incubation in buffer containing 0.5% Triton X-100 for 45 min at 4 °C and subsequent
sonication for 1 min. PKCα translocation was detected by Western blot.

**Matrigel Invasive Assay**—Matrigel was purchased from BD Biosciences and stored at −20 °C. After thawing at 4 °C overnight, the Matrigel was diluted in serum-free RPMI1640 medium. 50 μl of the suspension were evenly inoculated onto the upper chamber of a 6.5-mm transwell membrane and allowed to form a gel at 37 °C. The Matrigel invasive assay was carried out according to the manufacturer’s instructions. A 0.2-ml aliquot containing 200,000 cells was seeded on the upper chamber of the Matrigel-coated transwell filter (8 μm pore). The Dextrancoated charcoal-treated fetal bovine serum-containing medium was added to the lower chamber and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO2. Non-invading cells that remained on the upper surface of the filter were removed with cotton wool; the cells that appeared on the lower surface of the filter were fixed in 4% formaldehyde, stained with hematoxylin, and counted under a microscope. Four independent experiments were performed. In each experiment Matrigel invasion for each experimental group was measured in triplicate.

**Matrigel Adhesion Assay**—Matrigel adhesion assay was performed as described (27) with some modifications. LNCaP-FGC cells were suspended in serum-free medium containing 0.1% ethanol or 10 nM R1881. The cell suspension (2.5 × 10⁵ cells in 0.5 ml of medium/well) was added to 24 wells precoated with Matrigel and allowed to attach by incubation at 37 °C for 60 min. Unattached cells were removed by repeated washing with phosphate-buffered saline. Attached cells were stained with 0.5% crystal violet for 10 min. After washing with water, the stained cells were extracted with 0.25 ml of 10% acetic acid, and the absorbance of the dye extract was measured at 590 nm.

**Cell Proliferation Assays**—The cell number was assessed indirectly by staining with crystal violet dye. LNCaP-FGC cells were seeded in 96-well plates (density 5,000 cells/well) in 5% Dextran-coated charcoal-treated fetal bovine serum phenol red-free RPMI 1640 and cultured for 24 h. Cells were then stimulated with 10 nM R1881. After incubation, the medium was removed and cells were gently rinsed with phosphate-buffered saline. Cells were fixed with 4% formaldehyde for 30 min and stained with crystal violet (0.1% w/v in ddH2O). The excess dye was carefully washed away with ddH2O. The stained cells were lysed with 0.1% acetic acid. The absorbance was measured at 590 nm.

**Immunohistochemistry**—The prostate samples were obtained from patients who had prostate biopsy for cancer diagnosis and subsequently received radical prostatectomy. All patients were subjected to 1–2 weeks of neoadjuvant hormone therapy with cyproterone acetate prior to the prostatectomy. The immunohistochemistry experimental procedure was carried by standard biotin-avidin complex method as previously described (21).

**Statistical Analysis**—Statistical comparisons between groups were made by analysis of variance followed by Fisher’s post-hoc test; p < 0.05 was considered significant.

---

**RESULTS**

**Androgen Regulates Ezrin Expression in Human PCa Cells**—Our previous findings suggest that ezrin may have a regulatory role on androgen actions in prostate epithelial cells (15, 21). To explore this hypothesis, we first studied whether androgen directly regulates ezrin expression in human PCa cells. As shown in Fig. 1A, treatment with the synthetic androgen R1881 increases ezrin mRNA levels upon R1881 treatment (8 h) alone or in combination with androgen receptor antagonist bicalutamide (10 μM) or the protein synthesis inhibitor cycloheximide (CHX) (10 μg/ml). The average value for the control sample was set to 1. Each column represents the average ± S.D. of four independent replicates. * indicates statistically significant (p < 0.05) expression changes after R1881 treatment.

**Androgen Regulation of Ezrin Function**
androgen effects are indirect and dependent on de novo produced androgen-regulated genes (Fig. 1B). Western blot analysis of whole cell protein extracts showed that ezrin levels are increased after androgen treatment in a manner that corresponds to the changes seen in mRNA levels (Fig. 2). Also in line with the gene expression measurements, androgen-induced stimulation of ezrin protein levels was inhibited by treatment with bicalutamide (Fig. 2).

We next tested whether similar effects on ezrin expression could be observed in the prostate of cancer patients subjected to androgen ablation therapy (Fig. 2B). Ezrin levels were examined by immunohistochemistry in the prostate tissue of three patients obtained from prostate biopsies before and after radical prostatectomy. The patients were all diagnosed with PCa and subjected to neoadjuvant androgen ablation therapy using cyproterone acetate. One of the biopsy samples was not suitable for analysis. In the other two patient samples analyzed, ezrin expression decreased in PCa cells after androgen ablation therapy. Although a proper statistical analysis was not possible due to the small number of samples, our data suggest that ezrin expression is under androgen control in human prostate.

**Ezrin Actions Are Necessary for Androgen-induced PCa Cell Invasion**—Androgen ablation is known to reduce metastases in androgen-sensitive models of PCa (28). As the regulation of cell motility by ezrin is well documented (29, 30), we next wanted to explore its role in androgen-stimulated cell invasion. A Matrigel invasion assay was used to study androgen effects. As shown in Fig. 2C, LNCaP-FGC cells have little invasive capacity in the absence of androgen. The addition of R1881 significantly increased the number of cells migrating through the Matrigel. This effect was inhibited by bicalutamide. Interestingly, bicalutamide alone also enhanced cell invasion, although moderately. Because bicalutamide treatment alone is unable to stimulate ezrin expression, it is possible that ezrin up-regulation by androgen is an epiphenomenon, not directly related to the effects of the hormone on cell invasion. To rule out this possibility, we studied the invasiveness of LNCaP-FGC cells expressing a siRNA targeting human ezrin (Fig. 3). Very low expression levels of ezrin were detected in androgen-deprived cells. Androgen treatment induces ezrin expression in control cells, an effect that was severely reduced in ezrin siRNA-transfected cells (Fig. 3A). Cells expressing ezrin siRNA show a significant reduction of both basal and androgen-induced Matrigel invasion (Fig. 3B). These effects could not be explained by changes in cell adhesion, because no differences in the number of cells attached to Matrigel were detected in the presence or absence of androgen in either cells transfected with siRNA targeting ezrin or luciferase (Fig. 3C). Because ezrin, through the activation of PI(3) kinase, is involved in the regulation of kidney-derived epithelial cell line (LLC-PK1) survival (31), we next explored ezrin actions on LNCaP-FGC cell proliferation. As Fig. 3D shows, expression of ezrin siRNA has no significant effects on LNCaP-FGC cell proliferation.
Ezrin Overexpression Is Not Sufficient to Promote PCa Cell Invasiveness—The findings that induction of cell invasion by androgen correlates with increased ezrin protein levels and that a siRNA targeting ezrin blocks this effect suggest that increasing ezrin expression could be sufficient to promote invasion. We tested this hypothesis by transiently overexpressing WT-ezrin in LNCaP-FGC cells. As shown in Fig. 4, although transfection with a VSV-G-tagged ezrin expression vector increased the protein levels of ezrin to levels beyond those achieved upon androgen treatment (Fig. 4A), no effect on Matrigel invasion was observed in the absence of androgen treatment (Fig. 4B).

On the other hand, treatment of ezrin-overexpressing cells with R1881 significantly enhanced the effects of androgen on cell invasion, suggesting that additional actions of androgen are required to activate ezrin function.

Ezrin Threonine 567 and Tyrosine 353 Residues Are Required for Androgen-stimulated Invasion—The hallmark of ezrin activation is the phosphorylation of Thr-567 in the C-terminal domain of the protein. Phosphorylation of Thr-567 in the C-terminal domain of ezrin requires previous interaction with membrane phospholipids, specifically Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (32). Therefore, we analyzed the effects of neomycin, a drug that depletes the cell membrane of phosphoinositol lipids, on androgen-induced invasiveness. As shown in Fig. 5B, neomycin treatment caused a dramatic inhibition of androgen-induced Matrigel invasion. Treatment with LY294002, a PI(3) kinase inhibitor, has a similar effect. In contrast, the MEK inhibitor U0126 showed no effect.

We next evaluated the actions of androgen on ezrin phosphorylation. As shown in Fig. 5C, R1881 treatment of cells transfected with WT-ezrin resulted in phosphorylation of both Thr-567 and Tyr-353 residues. Phosphorylation of Thr-567 was first evident 2 h after treatment and increased steadily up to 20 h, the latest time point measured. Tyr-353 phosphorylation followed a similar pattern. In line with previous findings (Figs. 1 and 2), androgen-induced ezrin phosphorylation was inhibited by treatment with bicalutamide. No phosphorylated Thr-567 residues were found in cells transfected with T567A-ezrin mutant. Interestingly, androgen induction of Tyr-353 phosphorylation was also inhibited in these cells. On the other hand, in Y353F-
ezrin mutant-transfected cells androgen induction of Thr-567 phosphorylation was not affected. These results indicate that Thr-567 phosphorylation is a prerequisite for the phosphorylation of Tyr-353 upon androgen stimulation.

Ezrin Is Important for the Matrigel Invasion of Androgen-resistant Cell Lines—We studied the possibility of ezrin being also involved in the regulation of the invasive properties of androgen-resistant cell lines LNCaP-R and PC3. LNCaP-R is a subclone of the LNCaP-FGC cell line that proliferates independently of androgen despite expressing the androgen receptor (38, 39). Interestingly, in the absence of androgen LNCaP-R cells showed enhanced invasive properties when compared with LNCaP-FGC (Fig. 6A). However, LNCaP-R cells retain the ability to respond to androgen treatment by increasing Matrigel invasion. Forced overexpression of ezrin enhanced both basal and androgen-induced levels of Matrigel invasion, although only the latter in a statistically significant fashion. Similarly to LNCaP-FGC, the overexpression of T567A- or Y353F-ezrin mutants blocked the capacity of androgens to induced invasion of LNCaP-R cells.

Invasive properties of PC3 cells were insensitive to androgen treatment as expected from a cell line that does not express androgen receptors (Fig. 6B). Forced overexpression of ezrin

FIGURE 4. *Effects of ezrin overexpression in LNCaP-FGC cells.* A, Western blot analysis of ezrin expression in LNCaP-FGC cells transfected with VSV-G-tagged WT-ezrin or control plasmid. A representative result of four independently performed experiments is shown. B, Matrigel invasiveness of LNCaP-FGC cells transfected with VSV-G-tagged WT-ezrin or control plasmid with or without R1881 (10 nM) stimulation. Each column represents the average of four independent replicates measuring the number of cells that invade through Matrigel; error bars represent ± S.D. * indicates statistically significant (p < 0.05) regulation after R1881 treatment. # indicates significant (p < 0.01) differences between cells transfected with VSV-G-tagged WT-ezrin or control plasmid.

FIGURE 5. *Ezrin phosphorylation is required for androgen stimulation of cell invasion.* A, Matrigel invasion assay of LNCaP-FGC cells transfected with VSG-G-tagged Y353F and T567A-ezrin mutant plasmids. B, the effects of pharmacological inhibitors for PI(3) kinase (LY294002, 10 μM), PIP2 (neomycin, 10 μM), and MEK (U0126, 10 μM) on R1881(10 nM)-induced Matrigel invasion. C, time-dependent induction of ezrin phosphorylation by androgen in LNCaP-FGC cells transfected with wild-type ezrin plasmid, measured at Thr-567 and Tyr-353. In panels A and B, each column represents the average of four independent replicates measuring the number of cells invading through Matrigel, and the error bars represent ± S.D. * indicates statistically significant (p < 0.05) regulation after R1881 treatment. # indicates significant (p < 0.01) differences between cells transfected with VSV-G-tagged Y353F and T567A-ezrin mutants or control plasmid. & indicates statistically significant (p < 0.01) differences between cells treated with R1881 alone or in combination with LY294002 or neomycin. In panel C, a representative result of four independent experiments is shown.
doubled the Matrigel invasion activity of these cells, whereas significantly reduced invasive capacity was observed in cells expressing either the T567A- or Y353F-ezrin mutants. Taken together, these findings identify ezrin as a key regulator of PCa cell invasion for both androgen-sensitive and -insensitive cells.

PKC \( \alpha \) Mediates Androgen Effects on Ezrin Phosphorylation—Given the pivotal role of Thr-567 in ezrin actions (26, 37), we explored the possible mechanisms that could account for the phosphorylation of this residue upon androgen treatment. Previous results have shown that PKC \( \alpha \) can phosphorylate ezrin in vitro (37) and also has a proposed role in PCa progression (40, 41). Therefore, we measured PKC \( \alpha \) membrane translocation, the hallmark of its activation, upon androgen stimulation. Fig. 7A shows that androgen treatment of LNCaP-FGC cells induces the translocation of PKC \( \alpha \) to the cell membrane. This effect was insensitive to inhibition by bicalutamide. When ezrin content was analyzed in the same membrane fractions, we found increased ezrin recruitment to the cell membrane upon androgen treatment coinciding with PKC \( \alpha \) translocation. Interestingly, ezrin translocation to the cell membrane was almost completely inhibited by treatment with bicalutamide.

LNCaP-FGC cells treated either with the PKC \( \alpha \)-specific inhibitor Go6976 or with the less specific calphostin C failed to migrate through the Matrigel in response to androgen. In contrast, Y27632, an inhibitor of Rho-associated protein kinase (ROCK), another kinase that can phosphorylate ezrin in vitro, produced opposite effects (Fig. 7B). Moreover, PKC \( \alpha \) inhibition using Go6976 and calphostin C inhibited androgen stimulation of Thr-567 and Tyr-353 phosphorylation (Fig. 7C), supporting a role of PKC \( \alpha \) as an activator of ezrin in response to androgen. Similar effects were found when treating the cell with neomycin. Treatment with MEK or ROCK inhibitors had no effect on ezrin phosphorylation, in line with their lack of inhibitory action on androgen-induced Matrigel invasion. Interestingly, LY294002 had no effect on androgen-induced ezrin phosphorylation, indicating that PI(3) kinase activity is not required for these effects.

Previous reports indicate that PKC \( \alpha \) can interact with ezrin in the cell membrane after activation by phorbol esters and that PKC \( \alpha \)-associated ezrin is enriched in phosphorylated Thr-567 (26, 37). To study whether the association between ezrin and PKC \( \alpha \) is regulated by androgens, we analyzed whether endogenously expressed PKC \( \alpha \) co-immuno-precipitates with VSV-G-tagged ezrin. As shown in Fig. 6D, androgen promotes PKC \( \alpha \) association to WT-ezrin. The T567A-ezrin mutant failed to interact with PKC \( \alpha \) but the Y353F-ezrin mutant showed an enhanced association. As
Androgen-induced PKCa translocation was also insensitive to PP2 inhibition (Fig. 8C).

**DISCUSSION**

In this report, we describe a novel mechanism whereby androgen regulates the metastatic properties of PCa cells. We have shown that ezrin is an essential intracellular mediator of cell invasion in hormone-sensitive and hormone-resistant PCa cells. Androgen treatment induces ezrin expression and also causes ezrin phosphorylation in Thr-567 and Tyr-353 residues. Overexpression of ezrin variants with mutations on either one of these residues or reduction of ezrin expression by means of siRNA result in the inhibition of androgen-induced invasion. Androgen activation of ezrin comprises at least two steps, ezrin phosphorylation in Thr-567 mediated by PKCa and the subsequent phosphorylation in Tyr-353, which requires Src activity. The proposed mechanism is schematically presented in Fig. 9.

At least two different kinases, ROCK and PKCa, have been shown to phosphorylate ezrin at Thr-567 in vitro (37, 42). We have shown that androgen-induced Thr-567 phosphorylation is sensitive to PKCa, but not ROCK, inhibition. Moreover, androgen treatment activates PKCa and promotes the interaction between PKCa and ezrin in PCa cells. This interaction was abrogated by mutation of the Thr-567 phosphorylation site and was shown in Fig. 7E, androgen-induced association of PKCa to ezrin was inhibited by treatment with bicalutamide. The PKCa-specific inhibitor Go6976, and to a less extent calphostin C, previously demonstrated to inhibit Thr-567 phosphorylation reduced the PKCa/ezrin association. The PI(3) kinase inhibitor LY294002 had no effect.

Other studies have shown that androgen treatment induces Src tyrosine kinase activity and have demonstrated that ezrin can be a target of this kinase (26). Therefore, we next explored whether androgen induction of ezrin phosphorylation requires Src. As shown in Fig. 8A, the treatment with the specific Src inhibitor PP2 resulted in almost complete inhibition of ezrin Tyr-353 phosphorylation in response to androgen. PP2 treatment also inhibited androgen-induced Matrigel invasion (Fig. 8B) despite having no effect on hormonal induction of ezrin protein levels or its membrane translocation.

Androgen Regulation of Ezrin Function
that cell type- or signal-specific factors not yet characterized participate in the formation of the PKCα/H9251-ezrin complex. These factors could provide a different scaffold for such interactions in LNCaP-FGC as compared with MCF-7 cells. To clarify this matter a better understanding of how PKCα/H9251 is activated by androgens is necessary.

Androgen receptor blockage using bicalutamide induced PKCα activation to levels similar to those found in androgen-treated cells. We have also observed that bicalutamide treatment results in the promotion of cell invasion. The mechanisms used by bicalutamide to promote cell invasion in vitro are poorly understood. Bicalutamide is a non-steroidal compound that binds the ligand binding domain of the AR (45). In contrast to androgens, bicalutamide binding renders the AR transcriptionally inactive. This antagonistic effect relies on its capacity to recruit co-repressor complexes to the AR (46). In addition, inhibitory actions of bicalutamide on AR DNA binding activity have also been reported (47). Consequently, bicalutamide is considered to mainly antagonize the AR-mediated transcriptional (genomic) effects of androgens. On the other hand, the early events in the ligand-induced activation of the AR, which involves its nuclear translocation, are recapitulated by bicalutamide treatment (although at slower rates) (48). Therefore, it is plausible that both androgen and bicalutamide effects on PKCα activation rely on nongenomic, cytosolic actions of the AR (49).

In support of this hypothesis are the findings that bicalutamide as well as testosterone promote the activation of ERK (extracellular signal-regulated kinase) (50) in AR-expressing PC3 cells and that AKT (although not Src) is activated by bicalutamide in LNCaP-FGC cells (51, 52). Rapid elevation of intracellular levels of calcium and diacylglycerol upon androgen treatment have been previously described that could directly contribute to PKCα activation by R1881 (53). Speculatively, this could also be a mechanism contributing to bicalutamide activation of PKCα.

One cannot exclude that AR-independent mechanisms contribute to the actions of bicalutamide as well as R1881. In the case of androgens, those effects could be triggered by membrane-bound sex hormone binding globulin (SHBG). In addition, the existence of an independent transmembrane androgen receptor has been proposed, although such a molecule has not being identified yet. Because bicalutamide has low affinity for SHBG (45) and is structurally unrelated to androgens it is unlikely they share additional receptor molecules other than

---

3 A. Flores-Morales, unpublished observations.
the AR. The final answer of how bicalutamide activates PKCα would require the direct measurement of upstream signaling intermediaries such as calcium, phospholipase C, and PDK-1.

It is clear that androgens are more potent in promoting cell invasion than bicalutamide. The latter, despite being used in 1000-fold higher concentration than R1881, just managed to promote cell invasion at ~50% levels of those observed after androgen treatment. One of the reasons for these differences is that bicalutamide by itself has no effect on ezrin expression or phosphorylation. On the contrary, bicalutamide inhibits androgen-induced ezrin activation through its negative effects on ezrin membrane translocation and ezrin-PKC interaction. Because these events occur at the cell membrane it is possible that AR is translocated to the membrane upon androgen binding to trigger signaling events that result in ezrin activation. AR is known to form a complex with membrane-bound c-Src tyrosine kinase and promote its activation upon androgen, but not bicalutamide, binding (52, 54). Moreover, Src is known to physically associate with ezrin (26), which suggests the existence of a quaternary complex involving ezrin, Src, PKCα, and AR with regulatory actions on Pca cells. Indeed, our results show that treatment with the Src inhibitor PP2 results in reduced ezrin Tyr-353 phosphorylation as well as reduced cell invasion upon androgen treatment without affecting ezrin membrane translocation.

Previous studies have shown that T567A-ezrin mutant is poorly phosphorylated in Tyr-145 tyrosine (16, 55), a residue located in the N-terminal domain of the protein. Here, we demonstrate that the T567A-ezrin mutant is poorly phosphorylated in Tyr-353 in response to androgen, suggesting that tyrosine kinase activity toward this residue also requires the conformational changes induced by Thr-567 phosphorylation. Phosphorylation on Tyr-353 is of biological relevance. The overexpression of ezrin Y353F-ezrin mutant significantly reduced androgen effects on Matrigel invasion. Because Src inhibition reduces ezrin Tyr-353 phosphorylation, it is possible that this kinase is directly responsible for this event. Indeed, residues 145 and 477 have been shown to be phosphorylated by Src, and inhibition of this kinase severely impairs ezrin tyrosine phosphorylation (36). On the other hand, kinase assays have shown that Tyr-353 is poorly phosphorylated by Src in vitro (36), indicating that the Src role may be indirect, allowing additional tyrosine kinases, such as those activated by epidermal growth factor (35) and hepatic growth factor (34), to phosphorylate ezrin. Because androgen treatment is known to activate Src (52), it is likely that Tyr-145 and Tyr-477 are also phosphorylated in response to androgen treatment. Indeed, ezrin Tyr-477 is part of a positive feedback loop to enhance Src activation. This in turn results in the activation of focal adhesion kinase and the regulation of epithelial cell spreading (26). Future studies will tell whether focal adhesion kinase activation by androgens (56) also requires ezrin and the role of Tyr-477 in this process.

Previous studies have shown that ezrin Tyr-353 phosphorylation is required for the p85 regulatory subunit of PI(3) kinase to interact with ezrin (31), which can lead to the downstream activation of PI(3) kinase/AKT pathway. Our results indicate that PI(3) kinase activity is required for androgen-induced cell invasiveness and should be acting downstream of ezrin in LNCaP-FGC cells because its pharmacological inhibition does not interfere with ezrin phosphorylation or association with PKCα. This possibility is not unlikely, given the finding in other systems that activation of PKCα by PDK-1 is insensitive to PI(3) kinase inhibition (57). Alternatively, PI(3) kinase could control cell invasion through ezrin-independent mechanisms.

In summary, we have shown that ezrin is required for androgen-induced cell invasion and unveiled the mechanisms whereby androgen regulates ezrin functions. Ezrin is positioned at the cross-roads of multiple pathways regulating migration adhesion and proliferation in response to various factors known to influence Pca cell behavior (26, 37, 52). The understanding of ezrin function in prostate epithelial cells could potentially provide much needed novel pharmaceutical targets for the treatment of advanced prostate cancer.

Acknowledgments—We thank Dr. Staffan Strömblad for critical comments on the study and Dr. Monique Arpin for providing various ezrin constructs.

REFERENCES
1. Draisma, G., Boer, R., Otto, S. J., van der Crijnssen, I. W., Damhuis, R. A., Schroder, F. H., and de Koning, H. J. (2003) J. Natl. Cancer Inst. 95, 868–878
2. Thompson, I. M., Goodman, P. J., Tangen, C. M., Lucia, M. S., Miller, G. J., Ford, L. G., Lieber, M. M., Csepedes, R. D., Atkins, J. N., Lippman, S. M., Carlin, S. M., Ryan, A., Szczepanek, C. M., Crowley, J. J., and Colman, C. A., Jr. (2003) N. Engl. J. Med. 349, 215–224
3. Makinen, T., Tammela, T. L., Hakama, M., Stenman, U. H., Rannikko, S., Aro, J., Juusela, H., Maatannoten, L., and Auvrin, A. (2003) Clin. Cancer Res. 9, 2435–2439
4. Bubendorf, L., Schopfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T. C., and Mihatsch, M. J. (2000) Hum. Pathol. 31, 578–583
5. Bolla, M., Collette, L., Blank, L., Warde, P., Dubois, J. B., Mirimanoff, R. O., Storme, G., Bernier, J., Kuten, A., Sternberg, C., Mattelaer, I., Lopez-Torrecilla, J., Pfeffer, J. R., Lino Cutajar, C., Zurlo, A., and Pierart, M. (2002) Lancet 360, 103–106
6. Messing, E. M., Manola, J., Sarosdy, M., Wilding, G., Crawford, E. D., and Trump, D. (1999) N. Engl. J. Med. 341, 1781–1788
7. D’Amico, A. V., Manola, J., Loffredo, M., Renshaw, A. A., DellaCroce, A., and Kantoff, P. W. (2004) J. Urol. 172, 249–255
8. Cheville, J. C., Tindall, D., Boelter, C., Jenkins, R., Lohse, C. M., Pankratz, V. S., Sebo, T. J., Davis, B., and Blute, M. L. (2002) Cancer 95, 1028–1036
9. Schellhammer, P., Sharifi, R., Block, N., Soloway, M., Venner, P., Patterson, A. L., Sarosdy, M., Vogelzang, N., Jones, J., and Kolvenbag, G. (1995) Urology 45, 745–752
10. Edwards, J., and Bartlett, J. M. (2005) Br. J. Cancer 92, 1327–1335
11. Edwards, J., and Bartlett, J. M. (2005) Br. J. Urol. 92, 1320–1326
12. Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T. I., Isola, J., and Kallioniemi, O. P. (1995) Nat. Genet. 9, 401–406
13. Edwards, J., Krishna, N. S., Grigor, K. M., and Bartlett, J. M. (2003) Br. J. Cancer 89, 552–556
14. Taplin, M. E., Bubley, G. J., Ko, Y. J., Small, E. J., Upton, M., Rajeshkumar, B., and Balk, S. P. (1999) Cancer Res. 59, 2511–2515
15. Pang, S. T., Dillner, K., Wu, X., Pousette, A., Norstedt, G., and Flores-Morales, A. (2002) Endocrinology 143, 4897–4906
16. Gautreau, A., Louvard, D., and Arpin, M. (2002) Curr. Opin. Cell Biol. 14, 104–109
17. Khanna, C., Wan, X., Bose, S., Cassaday, R., Olomu, O., Mendoza, A., Yeung, C., Gorlick, R., Hewitt, S. M., and Helman, L. J. (2004) Nat. Med. 10, 182–186
Androgen Regulation of Ezrin Function

18. Yu, Y., Khan, J., Khanna, C., Helman, L., Meltzer, P. S., and Merlino, G. (2004) Nat. Med. 10, 175–181
19. Makitie, T., Carpen, O., Vaheri, A., and Kivela, T. (2001) Investig. Ophthalmol. Vis. Sci. 42, 2442–2449
20. Akisawa, N., Nishimori, I., Iwamura, T., Onishi, S., and Hollingsworth, M. A. (1999) Biochem. Biophys. Res. Commun. 258, 395–400
21. Pang, S. T., Fang, X., Valdston, A., Egevad, L., and Ekman, P. (2004) Urology 63, 609–612
22. Valdston, A., Fang, X., Pang, S. T., Nilsson, B., Ekman, P., and Egevad, L. (2005) Eur. Urol. 48, 852–857
23. Hasenson, M., Hartley-Asp, B., Kihlfrans, C., Lundin, A., Gustafsson, J. A., and Pousette, A. (1998) Cancer Res. 58, 1187–1192
24. Pang, S. T., Flores-Mora, A., Skoog, L., Chuan, Y. C., Nordsted, G., and Arpin, M. (2004) Oncol. Rep. 11, 1187–1192
25. Bonaccorsi, L., Carloni, V., Muratori, M., Salvadori, A., Giannini, A., Carini, M., Serio, M., Forti, G., and Baldi, E. (2000) Endocrinology 141, 3172–3182
26. Srivastava, J., Elliott, B. E., Louvard, D., and Arpin, M. (2005) Mol. Biol. Cell 16, 1481–1490
27. Zhang, M., Altujwijiri, S., and Yeh, S. (2004) Oncogene 23, 3080–3088
28. Pfitzenmaier, J., Quinn, J. E., Odman, A. M., Zhang, J., Keller, E. T., Vessella, R. L., and Corey, E. (2003) J. Bone Miner. Res. 18, 1882–1888
29. Martin, T. A., Harrison, G., Mansel, R. E., and Jiang, W. G. (2003) Crit. Rev. Oncol. Hematol. 46, 165–186
30. Curto, M., and McClatchey, A. I. (2004) Cancer Cell 5, 113–114
31. Gautreau, A., Pouillet, P., Louvard, D., and Arpin, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7300–7305
32. Fievet, B. T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D., and Arpin, M. (2004) J. Cell Biol. 164, 653–659
33. Gautreau, A., Louvard, D., and Arpin, M. (2000) J. Cell Biol. 150, 193–203
34. Crepaldi, T., Gautreau, A., Comoglio, P. M., Louvard, D., and Arpin, M. (1997) J. Cell Biol. 138, 423–434
35. Krieg, J., and Hunter, T. (1992) J. Biol. Chem. 267, 19258–19265
36. Heiska, L., and Carpen, O. (2005) J. Biol. Chem. 280, 10244–10252
37. Ng, T., Parsons, M., Hughes, W. E., Monypenny, J., Zicha, D., Gautreau, A., Arpin, M., Gschmeissner, S., Verveer, P. J., Bastiaens, P. I., and Parker, P. J. (2001) EMBO J. 20, 2723–2741
38. Mitchell, S., Abel, P., Ware, M., Stamp, G., and Lalani, E. (2000) BJU Int. 85, 932–944
39. Pang, S. T., Weng, W. H., Flores-Mora, A., Johansson, B., Pourian, M. R., Nilsson, P., Poussette, A., Larsson, C., and Norsted, G. (2006) Prostate 66, 157–172
40. Liu, B., Maher, R. J., De Jongcheere, I. P., Popat, R. U., Stojakovic, S., Hannun, Y. A., Porter, A. T., and Honn, K. V. (1997) Adv. Exp. Med. Biol. 400B, 707–718
41. Zhao, X., and Day, M. L. (2001) Urology 57, 860–865
42. Tran Quang, C., Gautreau, A., Arpin, M., and Treisman, R. (2000) EMBO J. 19, 4565–4576
43. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993) J. Biol. Chem. 268, 9194–9197
44. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) Biochim. Biophys. Res. Commun. 159, 548–553
45. Furr, B. J., and Tucker, H. (1996) Urology 47, Suppl. 1A, 13–25; discussion 29–32
46. Zhu, P., Baek, S. H., Bourk, E. M., Ohgi, K. A., Garcia-Bassets, I., Sanjo, H., Akira, S., Kotol, P. F., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. (2006) Cell 124, 615–629
47. Farla, P., Hersmus, R., Trapman, J., and Houtsmuller, A. B. (2005) J. Cell Sci. 118, Pt. 18, 4187–4198
48. Tyagi, R. K., Lavrovsky, Y., Ahn, S. C., Song, C. S., Chatterjee, B., and Roy, A. K. (2000) Mol. Endocrinol. 14, 1162–1174
49. Heinlein, C. A., and Chang, C. (2002) Mol. Endocrinol. 16, 2181–2187
50. Peterziel, H., Mink, S., Schonert, A., Becker, M., Klocker, H., and Cato, A. C. (1999) Oncogene 18, 6322–6329
51. Kang, H. Y., Cho, C. L., Huang, K. L., Wang, J. C., Hu, Y. C., Lin, H. K., Chang, C., and Huang, K. E. (2004) J. Bone Miner. Res. 19, 1181–1190
52. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000) Cancer Res. 60, 615–629
53. Lieberherr, M., and Grosse, B. (1994) J. Biol. Chem. 269, 7217–7223
54. Sun, M., Yang, L., Feldman, R. I., Sun, X. M., Bhalla, K. N., Nicosia, S. V., and Cheng, J. Q. (2003) J. Biol. Chem. 278, 42992–43000
55. Pula, G., Crosby, D., Baker, J., and Poole, A. W. (2005) J. Biol. Chem. 280, 7194–7205
56. Papakonstanti, E. A., Kampa, M., Castanas, E., and Stournaras, C. (2003) Mol. Endocrinol. 17, 870–881
57. Sonnenburg, E. D., Gao, T., and Newton, A. C. (2001) J. Biol. Chem. 276, 45289–45297