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A Rapid, Nongenomic, Signaling Pathway Regulates the Actin Reorganization Induced by Activation of Membrane Testosterone Receptors

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The human prostate cancer cell line LNCaP bears functional membrane testosterone receptors, which modify the actin cytoskeleton and increase the secretion of prostate-specific antigen (PSA) within minutes. Membrane steroid receptors are, indeed, a newly identified element of steroid action that is different from the classical intracellular sites. In the present work, using a nonpermeable analog of testosterone (testosterone-BSA), we investigated the signaling pathway that is triggered by the membrane testosterone receptors' activation and leads to actin cytoskeleton reorganization. We report that exposure of cells to testosterone-BSA resulted in phosphorylation of focal adhesion kinase (FAK), the association of FAK with the phosphatidylinositol-3 (PI-3) kinase, and the subsequent activation of the latter as well as the activation of the small guanosine triphosphatases Cdc42/Rac1. Pretreatment of cells with the specific PI-3 kinase inhibitor wortmannin abolished both the activation of the small guanosine triphosphatases and the alterations of actin cytoskeleton, whereas it did not affect the phosphorylation of FAK. These findings indicate that PI-3 kinase is activated downstream of FAK and upstream of Cdc42/Rac1, which subsequently regulate the actin organization. Moreover, wortmannin diminished the secretion of PSA, implying that the signaling events described above are responsible for the testosterone-BSA-induced PSA secretion. Our results are discussed under the prism of a possible implication of these membrane receptors in prostate cancer chemotherapy. (*Molecular Endocrinology* 17: 870–881, 2003)

THE ACTION OF steroid hormones and their biological effects were the subject of research a few decades ago and remain an important research area now (1–5). The classical, genomic action of steroid hormones includes binding to intracellular cognitive hormone receptors that share characteristics of nuclear transcription factors. After homo- or heterodimerization, steroid-bound receptors translocate to the nucleus and bind to specific DNA-steroid hormone-responsive elements, inducing or repressing a number of hormone-responsive genes. Each steroid receptor exhibits specific hormone- and DNA-binding domains, as well as specific moieties responsible for the binding of other accessory regulatory proteins. The effects of steroids in different cells occur after a given time lag, which represents the time necessary for the sequence: 1) hormone internalization; 2) binding to receptors; 3) dimerization; 4) nuclear translocation; 5) DNA binding; 6) activation/repression of genes; 7) transcription; 8) translation; 9) protein effects/secretion (6). However, in recent years, a number of reports indicate that steroids exert a number of effects in cells lacking classical receptors. In addition, in a number of reports, the effect of steroids was very rapid, occurring in minutes, a time lag noncompatible with the classical scheme of a nuclear receptor action (7, 8). These findings led to the identification of nonclassical estrogen, progesterone, and glucocorticoid binding elements in the plasma membrane, considered as new receptors for the steroid hormone family (8–11). Through binding to these sites, steroids could exert short-term effects, including calcium mobilization, secretion, and cytoskeleton modifications (12), regulated by the activation of signaling molecules.

Membrane binding sites for androgen were identified, in cells lacking (13–15) or presenting classical androgen receptors (16, 17), such as T lymphocytes, monocytes, and osteoblasts. The common element found for testosterone action in all these studies was the increase of free intracellular calcium (18, 19). Recently, we have reported the identification of membrane testosterone receptors on LNCaP human prostate cancer cells (20). Their activation results, within minutes, to a rearrangement of the actin cytoskeleton and to an increase of prostate-specific antigen (PSA) secretion to the culture medium. This later result requires a submembrane reorganization of the actin cytoskeleton, as the coincubation of cells with the actin-disrupting agent cytochalasin B reverts the effect (20).

Abbreviations: CA, Cyproterone-acetate; DDE, 4,4′-DDE 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene; DHT, dihydrotestosterone; FAK, focal adhesion kinase; GST, glutathione-*S*-transferase; GTPase, guanosine triphosphatase; PBD, p21-binding domain of PAK1; PI-3, phosphatidylinositol-3; PSA, prostate-specific antigen; TLC, thin layer chromatography.

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Actin cytoskeleton rearrangement, modifying cell-substratum adhesion, controls many cell functions (*e.g*. motility, division, and secretion) and is involved in a large number of human diseases (21–23). Indeed, focal adhesions are the places where the cellular membrane is attached to the substratum. Cell adhesion molecules consist of a network of cytoskeletal proteins that links the filamentous actin with the extracellular matrix (24). The maintenance of cell-matrix contact is of major significance for cell survival, whereas the dissolution of focal adhesions has been shown to precede apoptosis (25–28). In addition, the disassembly of stress fibers and focal adhesions are implicated in stimulating cell motility and invasion (29). Initial signals controlling these rearrangements include modification of focal adhesion kinase (FAK) activity, a protein tyrosine kinase that is activated by a variety of stimuli including integrins, growth factors, steroid hormones, cytokines, and neuropeptides (30–33). FAK also seems to play a role in tumor development because it has been shown that primary human cancer cells or cell lines overexpress the protein as well as its phosphorylated form (34–36).

FAK transmits the extracellular signals by activating downstream signaling molecules such as PI-3 kinase (37, 38), which has been implicated in cell survival and actin reorganization (39, 40). However, it has been shown that activation of PI-3 kinase may occur upstream from FAK phosphorylation (41, 42). PI-3 kinase phosphorylates inositol lipids at the 3′-position of the inositol ring generating the lipid products phosphatidylinositol-3-phosphate, phosphatidylinositol-3,4-diphosphate, and the phosphatidylinositol-3,4,5-triphosphate. These lipids are involved in the activation of the downstream effectors Akt/PKB or small guanosine triphosphatases (GTPases) Cdc42 and Rac1 (39). Akt/PKB, in turn, promotes cell survival and protects cells against cell death induced after detachment from extracellular matrix (43, 44). On the other hand, the activated, GTP-bound, Cdc42 and Rac lead to cell morphological changes through their effects on the actin cytoskeleton (45–47). More specifically, Cdc42 has been implicated in the formation of peripheral filopodia, Rac promotes cortical actin polymerization and cell ruffles, Rho stimulates the formation of stress fibers (29, 48), and all three GTPases regulate the formation of focal adhesion complexes (49). A variety of stimuli trigger the activation of key signaling molecules, including the GTP-binding proteins, protein kinases, or phosphoinositide kinases that subsequently regulate the dynamics of actin filaments (21, 50–53).

The aim of the present investigation was to analyze the mechanism of actin cytoskeleton modifications induced by membrane testosterone binding in LNCaP cells. We report that the pathway that transmits the signal from the testosterone-BSA to the actin cytoskeleton is through the FAK→PI-3 kinase→Cdc42/Rac1 cascade and that blockage of this cascade results in inhibition of both actin reorganization and PSA secretion.

RESULTS

Testosterone Membrane Binding Induces the Phosphorylation of FAK and Its Association with PI-3 Kinase

FAK activation by phosphorylation is the first element that transmits extracellular signals to downstream signaling molecules such as PI-3 kinase (37, 38), leading to actin reorganization (39, 40). We have therefore examined whether the active metabolite of testosterone, dihydrotestosterone (DHT), or the noninternalizable testosterone-BSA (10^{-7} M) could modify FAK phosphorylation. As shown in Fig. 1A the phosphorylation of FAK was significantly increased in both DHT- and testosterone-BSA-treated cells. However, FAK phosphorylation was induced to a lesser extent in DHT-treated cells compared with that in testosterone-BSA-treated cells.

It has been shown that FAK associates with a variety of adaptor or signal transduction molecules including the PI-3 kinase (37–40). We have examined whether such an association was induced by incubation of LNCaP cells with DHT or testosterone-BSA. As shown in Fig. 1B, p85 was coimmunoprecipitated with FAK in cells treated with DHT or testosterone-BSA, indicating that the activation of the membrane testosterone receptors resulted in the association of FAK with the p85 subunit of the PI-3 kinase. In this case too, significantly higher association was found when the nonpermeable testosterone analog testosterone-BSA was used, as compared with DHT.

PI-3 Kinase Is Activated Downstream of FAK by the Activation of Membrane Testosterone Receptors

PI-3 kinase is a signal transduction molecule that is activated upstream or downstream of FAK (38, 42) and influences the organization of actin cytoskeleton. To examine whether PI-3 kinase is activated by membrane testosterone receptors, an *in vitro* kinase assay was performed on antiphosphotyrosine immune complexes isolated from equal amounts of proteins of control and DHT or testosterone-BSA-treated cells. We found that the lipid kinase activity of PI-3 kinase (Fig. 2A) as well as the tyrosine phosphorylation of its p85 regulatory subunit (Fig. 2B) were significantly induced by androgens, while testosterone-BSA showed also, in this case, higher results as compared with DHT. This difference could be explained by the fact that testosterone-BSA acts solely on membrane receptors, while DHT freely penetrates the cell; thus, the extracellular concentration of DHT might be lower than that of testosterone-BSA. However, to exclude the possibility that

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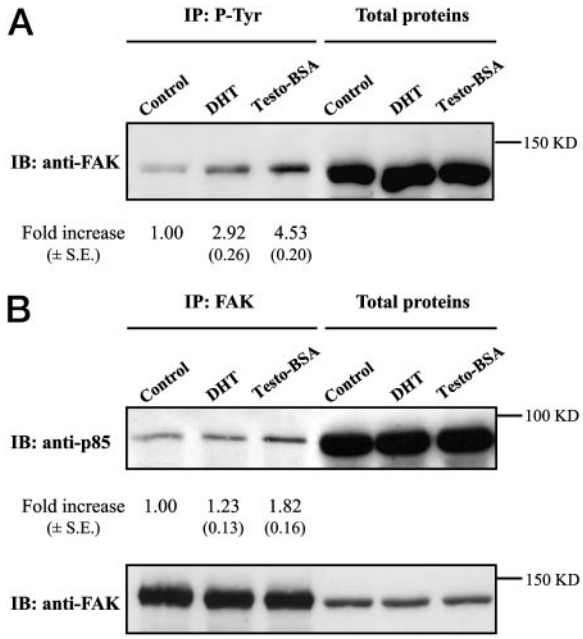

Fig. 1. Membrane Testosterone Receptors Trigger the Phosphorylation of FAK and Its Association with PI-3 Kinase

A, Cells were stimulated with DHT or testosterone-BSA for 10 min and lysed, and then equal amounts of proteins were immunoprecipitated (IP) with an antiphosphotyrosine antibody. Additionally, equal amounts of total lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The tyrosine-phosphorylated as well as the total amount of FAK were detected by immunoblotting (IB) with a specific anti-FAK antibody. The *number below each lane* indicates the fold phosphorylation of FAK, with that of untreated cells taken as 1. B, Cells were stimulated with DHT or testosterone-BSA and lysed and FAK was immunoprecipitated (IP) with a rabbit polyclonal anti-FAK antibody. Coimmunoprecipitated PI-3 kinase was detected by immunoblot (IB) with a rabbit polyclonal anti-PI-3 kinase (p85) antibody (*upper panel*). Stripping and reprobing of the nitrocellulose membrane confirmed that equal amounts of FAK protein were immunoprecipitated in each sample (*lower panel*). The *number below each lane* indicates the fold amount of PI-3 kinase coimmunoprecipitated with FAK, with that of untreated cells taken as 1. Each value represents the mean ± SE of three independent experiments.

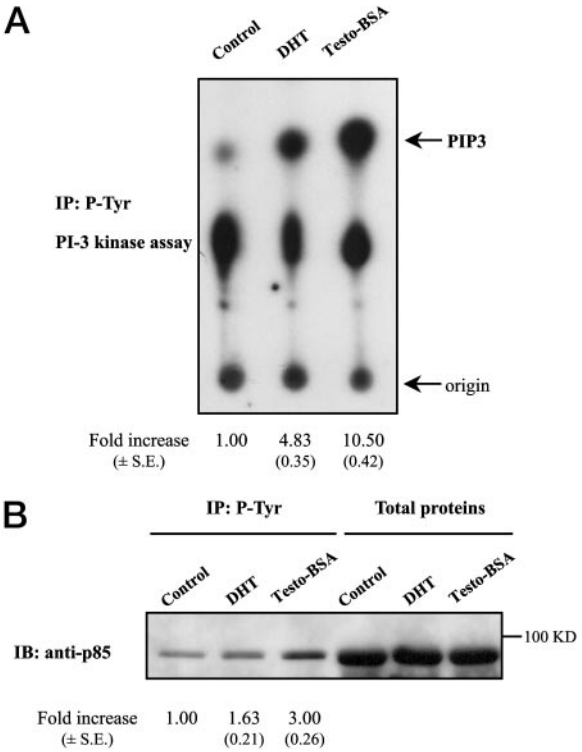

Fig. 2. Effect of DHT and Testosterone-BSA on PI-3 Kinase Activity

A, Cells were incubated for 10 min with DHT or testosterone-BSA. Equal amounts of proteins of cell lysates were immunoprecipitated with an antiphosphotyrosine antibody and subjected to an *in vitro* PI-3 kinase assay, as described in *Materials and Methods*, using phosphatidylinositol-4,5-bisphosphate (PIP2) as substrate. The reaction products were separated by TLC and visualized by autoradiography. The *number below each lane* indicates the fold amount of PIP3 product, with that of untreated cells taken as 1. B, The phosphorylation of the p85 regulatory subunit of PI-3 kinase that was immunoprecipitated in the kinase assay was assessed by immunoprecipitation (IP) with an antiphosphotyrosine antibody and immunoblotting (IB) with anti-PI-3 kinase (p85) antibody. The *number below each lane* indicates the fold phosphorylation of p85, with that of untreated cells taken as 1. Each value represents the mean ± SE of three independent experiments. PIP3, Phosphatidylinositol-3,4,5-trisphosphate.

testosterone-BSA induces the activation of the above signaling molecules by its binding to classical androgen receptors, which could be localized to cell membrane, we examined whether androgen antagonists block the rapid phosphorylation of FAK or p85. For this, we performed experiments in the presence of the nonsteroidal antiandrogen flutamide, which exerts an inhibitory action on classical androgen receptor-mediated effects. As shown in Fig. 3A, instead of being attenuated, the phosphorylation of FAK (*upper panel*) as well as p85 (*lower panel*) was increased by testosterone-BSA in the presence of flutamide. The same results were obtained when flutamide was replaced by the steroidal antitestosterone, cyproterone-acetate (CA), or the nonsteroidal DDE [4,4′-DDE 1, 1-dichloro-2,2-bis(4-chlorophenyl)ethylene] (Fig. 3B). The fact that three different antiandrogens, derived from different classes of antiandrogen families, do not antagonize the effect of testosterone-BSA implies that the signaling cascade events described above are mediated through novel membrane receptors. To analyze further the specificity of testosterone-BSA effects on the phosphorylation of FAK and the p85 regulatory subunit of PI-3 kinase, we performed experiments with increasing concentrations of testosterone-BSA and DHT (Fig. 4). As shown in Fig. 4A, 10-min incubation of LNCaP cells with 10^{-10} to 10^{-6} M of testosterone-BSA induced a dose-dependent phosphorylation of FAK and p85 that reached

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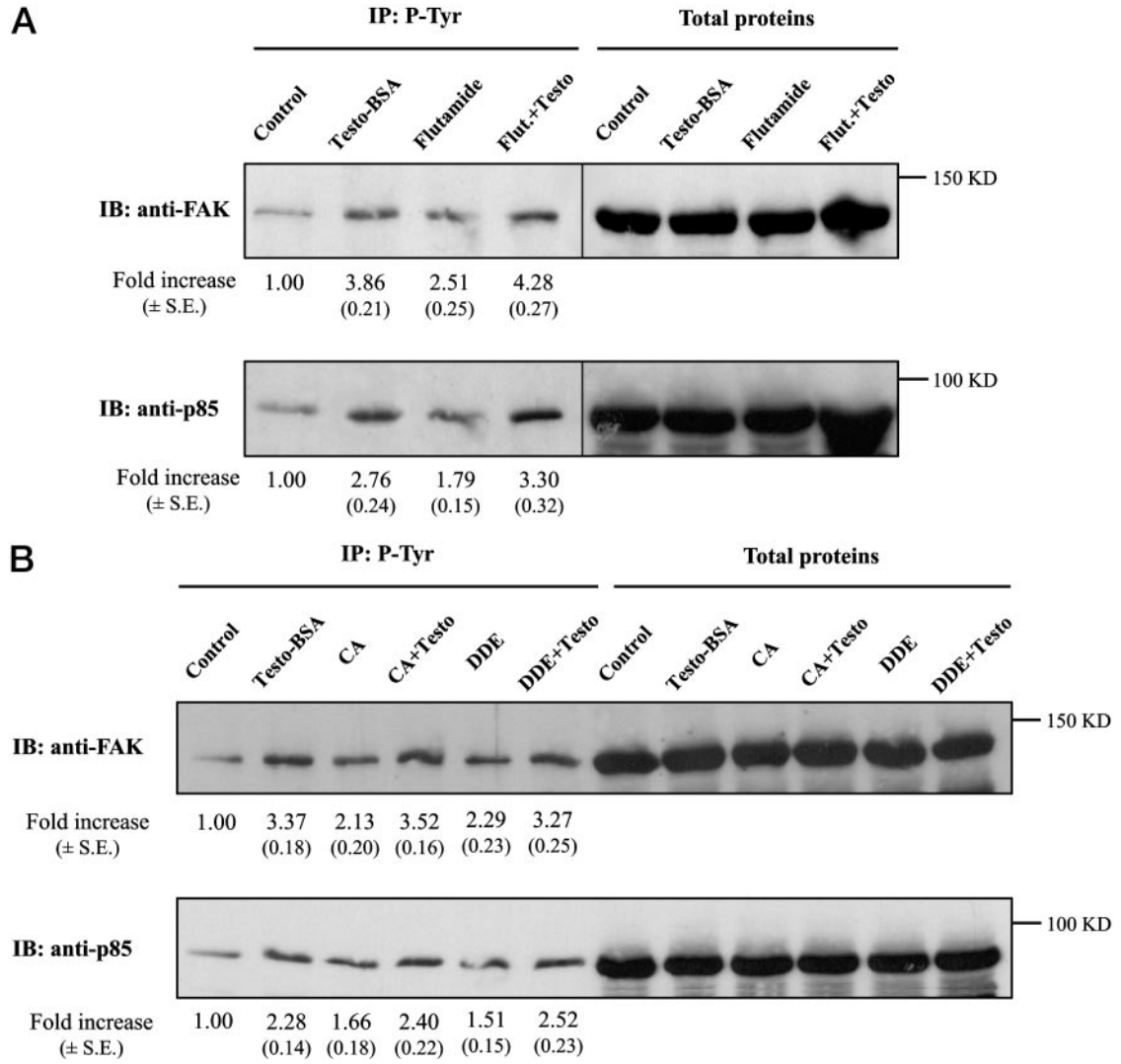

Fig. 3. Novel Membrane Testosterone Receptors Trigger the Signaling Cascade in LNCaP Cells

LNCaP cells were incubated with 10^{-7} M testosterone-BSA in the absence or in the presence of 10^{-6} M nonsteroidal antiandrogen flutamide (A), the steroidal antiandrogen CA, or the nonsteroidal DDE (B), for 10 min. Cells were lysed and then equal amount of proteins were immunoprecipitated (IP) with an antiphosphotyrosine antibody. Additionally, equal amount of total lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The tyrosine-phosphorylated as well as the total amount of FAK (*upper panel*) or p85 (*lower panel*) was detected by immunoblotting (IB) with a specific anti-FAK or anti-p85 antibody, respectively. The *number below each lane* indicates the fold phosphorylation of FAK or p85, with that of untreated cells taken as 1. Each value represents the mean ± SE of three independent experiments.

the plateau at 10^{-6} M. The EC_{50} for FAK phosphorylation was determined to be 1.98×10^{-8} M and for p85 phosphorylation 2.55×10^{-8} M testosterone-BSA. When the same experiment was performed using DHT (Fig. 4B), the EC_{50} for FAK phosphorylation was determined to be 7.54×10^{-7} M and for p85 phosphorylation 6.79×10^{-7} M DHT, indicating that a higher extracellular concentration of DHT than that of testosterone-BSA is needed to obtain the same percent of FAK and p85 activation. These results confirm our above hypothesis according to which testosterone-BSA is more effective than DHT because testosterone-BSA acts solely on membrane receptors while DHT penetrates the cell, thereby decreasing its extracellular concentration.

To determine whether PI-3 kinase is an effector of FAK, LNCaP cells were pretreated with the specific PI-3 kinase inhibitor wortmannin and then treated with DHT or testosterone-BSA. As shown in Fig. 5A, the phosphorylation of FAK remained unchanged by wortmannin, indicating that PI-3 kinase is activated downstream of FAK. On the other hand, pretreatment of cells with wortmannin abolished the formation of $PI(3,4,5)P_3$, confirming the inhibition of PI-3 kinase activity (Fig. 5B). The phosphorylation of the p85 subunit was not inhibited (Fig. 5C) because wortmannin interacts with the p110 catalytic

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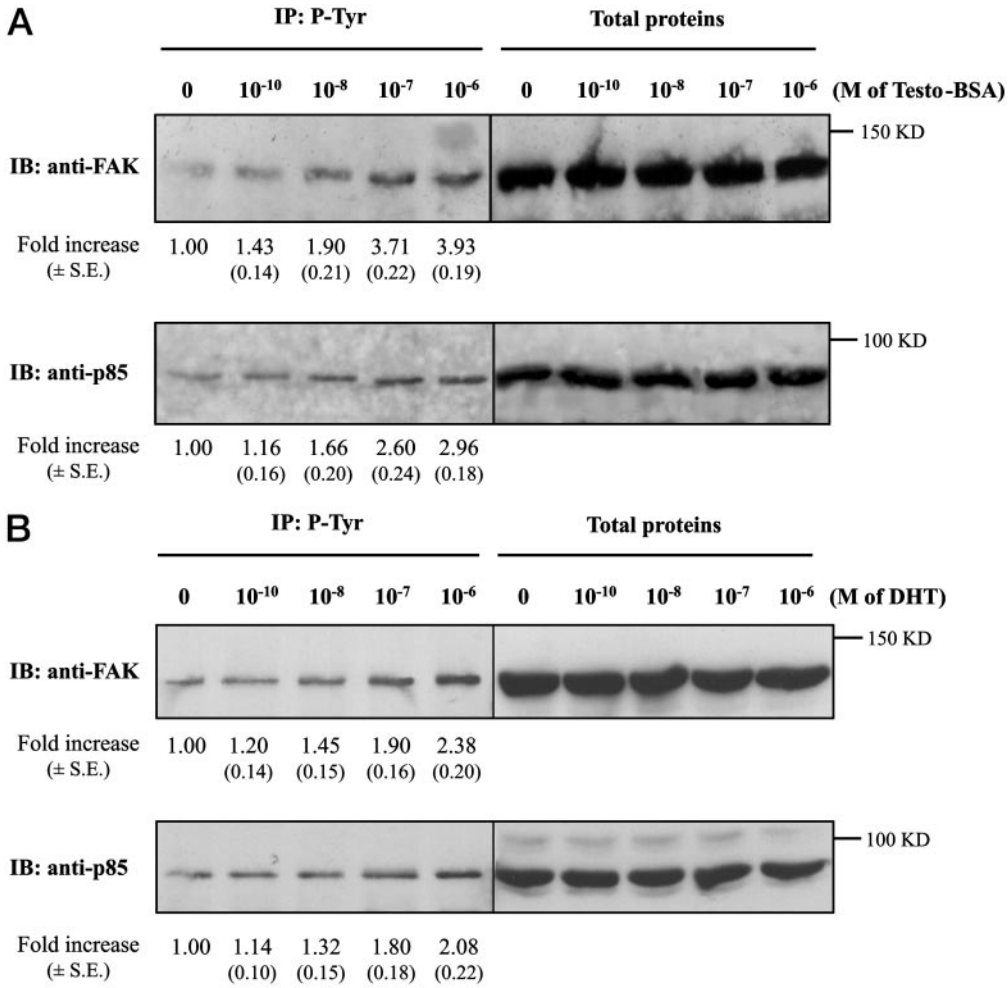

Fig. 4. Dose-Dependent Effects of Testosterone-BSA and DHT on FAK and p85 Phosphorylation

Cells were incubated for 10 min with the indicated concentrations of testosterone-BSA (A) or DHT (B), and then cells were lysed and equal amount of proteins were immunoprecipitated (IP) with an antiphosphotyrosine antibody. Additionally, equal amounts of total lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The tyrosine-phosphorylated as well as the total amount of FAK (*upper panel*) or p85 (*lower panel*) was detected by immunoblotting (IB) with a specific anti-FAK or anti-p85 antibody, respectively. The *number below each lane* indicates the fold phosphorylation of FAK or p85, with that of untreated cells taken as 1. Each value represents the mean ± SE of three independent experiments.

subunit of PI-3 kinase (54) and would not necessarily be expected to affect tyrosine phosphorylation of the p85 regulatory subunit. Furthermore, as shown in Figs. 1B and 2B, FAK associates and phosphorylates the p85 subunit and thus wortmannin would not be expected to inhibit this phosphorylation since FAK was activated upstream and independently of PI-3 kinase activity.

PI-3 Kinase Induces the Activation of Cdc42/Rac1 and Actin Reorganization in Response to the Activation of Membrane Testosterone Receptors

Because the small GTPases are implicated in actin reorganization (29), we examined whether Cdc42 and Rac1 are activated in response to DHT or testosterone-BSA. Equal volumes of cell lysates from control and DHT or testosterone-BSA-treated cells were affinity precipitated with a glutathione-*S*-transferase (GST)-fusion protein corresponding to the p21-binding domain of PAK1 (GST-PBD) that specifically binds to and precipitates the Cdc42-GTP and Rac1-GTP (55). The presence of each GTPase was assessed with specific antibodies. To normalize the results, equal volumes of cell extracts were also analyzed by immunoblotting for total amounts of Cdc42 and Rac1. As shown in Fig. 6A, the ratio of GTP-Cdc42 or GTP-Rac1 to total Cdc42 or Rac1, which represents the normalized amounts of the active small GTPases, was significantly increased in cells exposed to DHT or to testosterone-BSA.

To examine whether Cdc42 and Rac1 are downstream effectors of PI-3 kinase, the activated forms of the small GTPases were determined in cells that had

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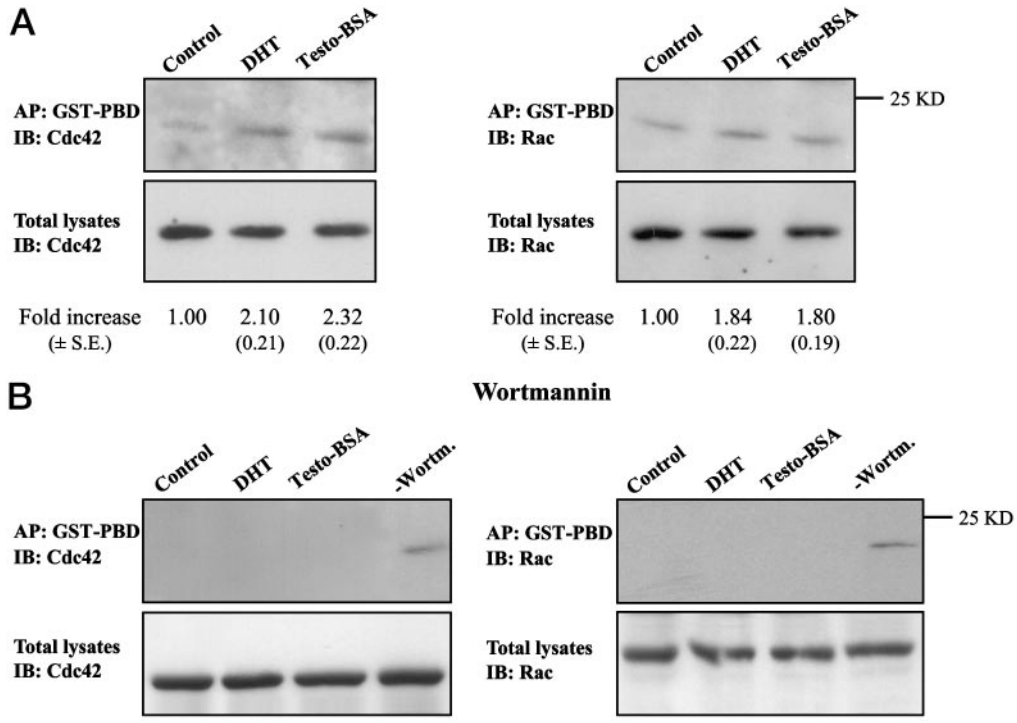

Fig. 6. Cdc42 and Rac1 Are Activated Downstream of the PI-3 Kinase in Response to DHT or to Testosterone-BSA

A, Equal volume of cell lysates from untreated, DHT-, or testosterone-BSA-treated cells were affinity precipitated (AP) with GTP-PBD bound to glutathione-agarose beads. Precipitated GTP-Cdc42 or GTP-Rac1 was detected by immunoblot (IB) with anti-Cdc42 or anti-Rac1 antibody, respectively (*upper two panels*). Equal volumes of total lysates from untreated and DHT- or testosterone-BSA-treated cells were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted (IB) with monoclonal anti-Cdc42 or anti-Rac1 antibody, respectively (*lower two panels*). The *number below each lane* indicates the normalized fold activation of Cdc42 or Rac1, with that of untreated cells taken as 1. Each value represents the mean ± SE of three independent experiments. B, Cells were pretreated with wortmannin for 30 min and stimulated with DHT or testosterone-BSA after which the activation of Cdc42 or Rac1 was examined as described in panel A. Similar results were obtained in three independent experiments.

terone receptors and leads to actin redistribution seems to be responsible for the increase of PSA secretion.

DISCUSSION

The recent discovery of steroid hormone membrane receptors opened a new field in the elucidation of steroid action. Indeed, the activation of membrane sites could explain intriguing results of steroid action, which could not be explained by their genomic effects. Membrane binding sites for all steroid hormones have been detected in a number of cell types (7–12, 18, 19), and all reports indicate that, as a result of their activation, an increased extracellular Ca^{2+} flux is observed. Testosterone receptors have been found in cell types bearing (16, 17) or not bearing (13–15) classical intracellular receptors. Recently, we have reported for the first time, the existence of noninternalizable membrane testosterone receptors on LNCaP human prostate cancer cells (20). The activation of these sites results in a reorganization of the actin cytoskeleton and an increased PSA secretion. In addition, we have found that the expression of these sites is restrained in cells expressing a malignant phenotype, *i.e*. benign prostate hyperplasia, and normal peritumoral cells do not express these sites (Stathopoulos, E. N., C. Dambaki, M. Kampa, G. S. Delides, and E. Castanas, submitted).

The main purpose of the present work was to investigate the signaling cascade leading from activation of membrane testosterone receptors, to actin cytoskeleton reorganization. For this, we have examined the phosphorylation and activation of FAK after short incubation of cells with the nonpermealizable testosterone analog testosterone-BSA. FAK is a nonreceptor tyrosine kinase that is localized in focal adhesions. In addition to integrins that mediate the adhesion of cells to extracellular matrix, a variety of extracellular stimuli including growth factors, steroid hormones, cytokines, and neuropeptides induce the tyrosine phosphorylation and activation of FAK (30–33). Its activation requires an intact actin cytoskeleton (56), whereas its downstream cascades can lead to actin cytoskeleton reorganization

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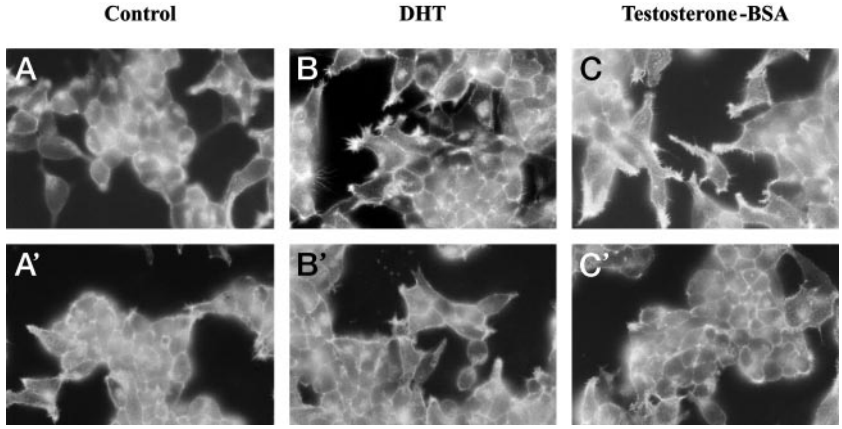

Fig. 7. The Redistribution of Actin Filaments Induced by DHT or Testosterone-BSA Depends on PI-3 Kinase Activity

Cells were grown on poly-L-lysine coated glass coverslips, pretreated with wortmannin (A′, B′, C′) or with its vehicle (A, B, C) and then incubated for 10 min with DHT or testosterone-BSA. The redistribution of filamentous actin was determined with rhodamine-phalloidin staining by immunofluorescence microscopy. Magnification, ×400. Similar results were obtained in three independent experiments.

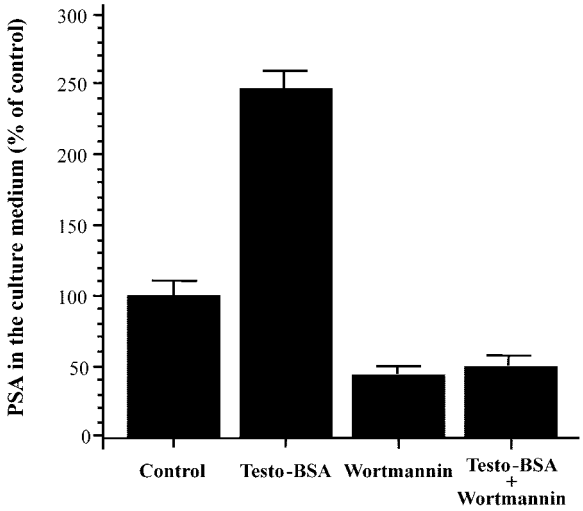

Fig. 8. Effect of Wortmannin on PSA Secretion

Cells were pretreated with wortmannin or its vehicle for 30 min and then stimulated with 10^{-7} M testosterone-BSA for 10 min. Medium was then preceded for PSA determination as described in *Materials and Methods*. Results are presented as a percentage of the basal (control) secretion. Mean ± SEM of three independent experiments performed in triplicate.

(30, 31). FAK also seems to play a role in tumor development since it has been shown that primary human cancer cells or cell lines overexpress the protein as well as its phosphorylated form (34–36). Indeed, FAK is thought to be a potential oncogene since it has been implicated in the progression of cancer to invasion and metastasis. In particular, FAK was found to be overexpressed and to be highly activated in tumorogenic PC-3 cells as well as in prostate cancer tissues from patients with metastasis, whereas in LNCaP cells that have a lower tumorigenic ability, FAK was observed to be much lower (34). Testosterone-BSA induced a significant increase of FAK phosphorylation, association of FAK with PI-3 kinase, and the activation of the latter (Figs. 1 and 2). Association of FAK with the PI-3 kinase has been shown to result in PI-3 kinase activation (37, 38), which is necessary for triggering downstream signaling pathways. In addition, this association function to recruit PI-3 kinase to focal contacts resulted, in other cell systems, in stimulated cell migration (57). However, according to published data, PI-3 kinase activity is required for FAK activation and cell migration (58–60). Using the specific PI-3 kinase inhibitor wortmannin, we found that PI-3 kinase is activated downstream of FAK and upstream of the small GTPases, Cdc42 and Rac1, in response to testosterone-BSA (Figs. 5 and 6).

It is known that PI-3 kinase activation results in actin cytoskeleton reorganization, through activation of the downstream effectors Cdc42 and Rac1, and such reorganization is involved in malignant transformation, cell migration, and cancer invasion (29, 40, 61). More specifically, the activation of Cdc42 leads to filopodia formation, whereas activated Rac1 promotes the formation of membrane ruffles and lamellipodia. These structures are observed in locomoting cells (29), and since migration is involved in the expression of the invasive phenotype, the small GTPases are considered important factors that influence the invasiveness of tumor cells *in vivo*. However, the role of the small GTPases to cell response seems to depend on cell type. In particular, it has been shown that activated Rac promotes migration and invasion of T lymphoma cells (62, 63) but inhibits the migration and invasion of epithelial cells (59, 64). Additionally, the Rho GTPases have been implicated in apoptosis induced by cytotoxic T lymphocytes and Fas through their effects on the actin cytoskeleton (47). Cdc42 and Rac have been also shown to regulate vesicle trafficking (65–67),

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whereas their activity may be required for the transport of proteolytic enzymes and receptors to the membrane of the invading cells (68). The results obtained in our system are consistent with these findings because wortmannin inhibited the activation of Cdc42/Rac1 (Fig. 6) and was followed by inhibition of PSA secretion (Fig. 8).

Our results show that testosterone-BSA induced the phosphorylation of FAK, which was followed by the activation of PI-3 kinase, and this was accompanied by the activation of the Cdc42/Rac1, resulting in the redistribution of peripheral actin. Pretreatment of LNCaP cells with the specific PI-3 kinase inhibitor wortmannin resulted in inhibition of both the reorganization of the actin filaments (Fig. 7) and the testosterone-BSA-induced PSA secretion (Fig. 8), indicating that the organization of actin cytoskeleton could account for the regulation of the PSA secretion. This hypothesis was also supported by our previously published data according to which the actin-disrupting agent cytochalasin B, abolished the secretion of PSA (20). The above results imply that testosterone receptors located on cell membranes of LNCaP cells may induce the secretion of PSA by activating key signaling molecules in a hierarchy of FAK→PI-3 kinase→Cdc42/Rac1→actin reorganization. However, an additional signaling cascade linking the PI-3 kinase with the secretion of PSA, independently of actin, could not be excluded although the mechanism by which this might occur is not yet understood. The fact that testosterone was less active than testosterone-BSA conjugate (Figs. 1 and 2), as well as the findings showing that a number of different androgen receptor antagonists did not inhibit the phosphorylation of FAK or p85 (Fig. 3), further indicates that this signaling cascade might be specific for the activation of testosterone membrane-binding sites. The specificity of these membrane testosterone-binding sites has been addressed in our previous work (20). Indeed, we have reported that estradiol and progesterone are 10^4 and 10^2 times less active, respectively, than testosterone in displacing [^{3}H]testosterone in isolated membranes of LNCaP cells. On the other hand, the K_D of testosterone on membrane sites was found to be 10.9 nM, a result comparable to the effect of testosterone-BSA on FAK and p85 phosphorylation (Fig. 4A).

Under the current state of knowledge, the physiological significance of our findings remains speculative. First, the effects of membrane testosterone receptors stimulation and the actin cytoskeleton reorganization are expressed within minutes. In addition, it appears, from the results presented in Fig. 6, that a main effect of the cytoskeletal reorganization might be the secretion of stocked PSA from the cell. Indeed, this secretion, also resulting within minutes, is inhibited by the addition of wortmannin (Fig. 8). Finally, chronic stimulation of membrane testosterone receptors results in an inhibition of cell growth (Kogia, E., and E. Castanas, unpublished observations), indicating that this signaling cascade activation and actin reorganization might be related to cell growth inhibition, as was previously reported for opioid drugs (69). It should be interesting therefore, to explore further this effect as a possible new target of prostate cancer chemotherapy, a possibility currently investigated by our group.

MATERIALS AND METHODS

Materials

Rhodamine-phalloidin and Slow Fade Antifade kit were from Molecular Probes, Inc. (Eugene, OR). Monoclonal antibodies for Cdc42 and antiphosphotyrosine (PY20) as well as polyclonal antibody for FAK (rabbit) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-PI-3 kinase(p85) antibody, monoclonal anti-Rac, and Cdc42/Rac1 activation assay kit [including GST-fusion of the PBD (amino acids 67–150) bound to glutathione-Agarose and lysis/wash buffer] were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phosphatidylinositol-4,5-bisphosphate (from bovine brain) and wortmannin were obtained from Sigma (St. Louis, MO). Silica gel 60 sheets were from Merck & Co., Inc. (Poole, Dorset, UK) Enhanced chemiluminescence Western blotting kit, protein A-Sepharose beads, and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech (Piscataway, NJ). Testosterone-3-(O-carboxymethyl)oxine-BSA (named testosterone-BSA), flutamide, CA, and DDE were obtained from Sigma (St. Louis, MO). All other chemicals were obtained from usual commercial sources at the highest grade available.

Testosterone-3-(O-carboxymethyl)oxine-BSA (named testosterone-BSA) was dissolved in culture medium at twice the concentration indicated in each experiment. Before each experiment, this stock solution of testosterone-BSA was treated with a solution of charcoal (50 mg/ml) and dextran (0.05 mg/ml) for 30 min at room temperature, centrifuged at 3000 $\times$ *g* for 15 min and passed through a 0.22 μm, to remove any potential contamination with free steroid. A specific assay of treated testosterone-BSA (AxSim testosterone assay, Abbott Laboratories, Chicago, IL) did not reveal the presence of any free testosterone. This treated testosterone-BSA was subsequently used throughout all studies.

Cells and Culture Conditions

The LNCaP cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 C in a humidified atmosphere of 5% CO_2 in air. They were subcultured once a week and incubated in serum-free medium for 24 h before any experiment. When indicated, the specific PI-3 kinase inhibitor wortmannin was used in a concentration of 100 nM, and it was added 30 min before to the addition of 10^{-7} M DHT or testosterone-BSA.

Immunoprecipitation, Kinase Assay, and Immunoblotting Analysis

Testosterone-BSA or DHT-treated, as well as untreated (control), cells were washed three times with ice-cold PBS and suspended in cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris (pH 7.4), and 137 mM NaCl, supplemented with protease and phosphatase inhibitors. Cleared lysates were preadsorbed with protein A-Sepharose for 1 h at 4 C and centrifuged, and the supernatants (equal amounts of protein) were subjected to immunoprecipitation using the indicated antibodies and the protein A-Sepharose beads.

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The lipid kinase activity of PI-3 kinase was measured by the method of Auger *et al.* (70) with minor modifications. Protein A-Sepharose beads containing immunoprecipitated phosphotyrosine proteins were washed three times with Buffer A [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40, 0.1 mM Na_3VO_4], three times with 5 mM LiCl in 0.1 M Tris (pH 7.4), and twice with TNE (10 mM Tris, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.1 mM Na_3VO_4). The immunoprecipitates were then resuspended in TNE, and the PI-3 kinase activity was assayed using 0.2 mg/ml phosphatidylinositol-4,5-bisphosphate as a substrate, in the presence of 58 μM ATP, 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol), and 14 mM $MgCl_2$, for 10 min at 37 C. The reaction was stopped by the addition of 1 M HCl and methanol/chloroform (1:1). After mixing vigorously and centrifuging to separate the phases, the lipids in the organic lower phase were separated by thin layer chromatography (TLC) on oxalated silica gel 60 sheets, as described (71). Chromatographed lipids were also visualized by iodine staining and compared with the migration of known standards.

For immunoblot analysis, the cell lysates or the immunoprecipitates were suspended in Laemmli's sample buffer and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6; 137 mM NaCl; 0.05% Tween-20) for 1 h at room temperature. Antibody solutions (in TBS-T containing 5% nonfat dry milk) were added overnight at 4 C (first antibody) and for 1 h (second horseradish peroxidase-coupled antibody). Blots were developed using the enhanced chemiluminescence system, and the band intensities were quantitated by PC-based image analysis (Image Analysis, Inc., Ontario, Canada).

Affinity Precipitation

Affinity precipitation with GST-PBD was performed using an assay based on the method of Benard *et al.* (55). Cells were lysed in Mg^{2+} lysis buffer that was provided by the assay kit (Upstate Biotechnology, Inc.), were mixed with 8 μg GST-PBD bound to glutathione-Agarose and incubated for 1 h at 4 C Precipitates were washed three times with Mg^{2+} lysis buffer and suspended in Laemmli's sample buffer. Proteins were separated by 11% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-Cdc42 or anti-Rac antibody.

Immunofluorescence Microscopy

The procedure of cell fixation and direct fluorescence staining of microfilaments by rhodamine-phalloidin included incubation of cells with 3.7% formaldehyde, followed by a short incubation with acetone at −20 C. The cells were then incubated for 40 min at room temperature with rhodamine-phalloidin to stain the filamentous actin (72). Slides were mounted using the Slow Fade Antifade kit. All specimens were examined with a BH-2 microscope (Olympus Corp., Lake Success, NY) equipped with epifluorescence illumination. Micrographs were photographed with a 35-mm (C-35AD-4) camera and Kodak P3200 black and white films (Eastman Kodak Co., Rochester, NY).

Measurement of Androgen-Induced PSA Secretion by LNCaP Cells

LNCaP cells were seeded in 24-well plates, at an initial density of 30,000 cells per well, and cultured for 48 h in order to achieve maximum attachment. Thereafter, culture medium was replaced with serum-free medium, and culture was followed for an additional 24 h. Androgens (DHT, testosterone-BSA; 10^{-7} M) were then added for 10 min, the medium was collected and centrifuged for 10 min at 1500 rpm, and total PSA was measured in the supernatant in an AxCim (Abbott Laboratories), using the corresponding kit from the same source, and normalized according to the total number of cells. Cell number was assayed using the tetrazolium salt assay (73).

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