Matriptase activation and shedding with HAI-1 is induced by steroid sex hormones in human prostate cancer cells, but not in breast cancer cells

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Submitted 14 July 2005; accepted in final form 5 February 2006

Matriptase and its inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1), have been implicated in carcinoma onset and malignant progression. However, the pathological mechanisms of matriptase activation are not defined. Steroid sex hormones play crucial roles in prostate and breast cancer. Therefore, we investigated the questions of whether and how steroid sex hormones regulate matriptase activation in these cancer cells. Treatment of cells with 17β-estradiol had no effect on activation of matriptase in hormone-starved breast cancer cells, in part due to their high constitutive level of activated matriptase. In striking contrast, very low levels of activated matriptase were detected in hormone-starved lymph node prostatic adenocarcinoma (LNCaP) cells. Robust activation of matriptase was observed as early as 6 h after exposure of these cells to 5α-dihydrotestosterone (DHT). Activation of matriptase was closely followed by shedding of the activated matriptase with >90% of total activated matriptase present in the culture media 24 h after DHT treatment. Activated matriptase was shed in a complex with HAI-1 and may result from simultaneously proteolytic cleavages of both membrane-bound proteins. Latent matriptase and free HAI-1 were also shed into culture media. As a result of shedding, the cellular levels of matriptase and HAI-1 were significantly reduced 24 h after exposure to DHT. DHT-induced matriptase activation and shedding were significantly inhibited by the androgen antagonist bicalutamide, by the RNA transcription inhibitor actinomycin D, and by the protein synthesis inhibitor cycloheximide. These results suggest that in LNCaP cells, androgen induces matriptase activation via the androgen receptor, and requires transcription and protein synthesis.

androgen; hepatocyte growth factor activator inhibitor-1

MATRIPTASE and its inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1), represent a cognate pair, a cell surface proteolytic enzyme and its inhibitor (29). Both are broadly coexpressed by epithelial elements in most epithelium-containing tissues (18, 45). Matriptase, a type 2 transmembrane serine protease (40, 54), contains a COOH-terminal serine protease domain and several noncatalytic domains, including a sperm protein-enteroxinase-agrin module, two tandem C1r/s-Uegf-Bone morphogenetic protein-1 domains, and four tandem low-density lipoprotein (LDL) receptor class A domains (19, 30, 57, 59). Matriptase was shown to play an important role in epidermal barrier function, terminal epidermal differentiation, hair follicle development and thymic homeostasis in matriptase knockout mice (32). Some of these functions may result from its role in the maturation of pro-filaggrin (34), a marker for the terminal differentiation of keratinocytes. The protease is expressed by a variety of human tumors of epithelial origins, including breast, ovarian, uterine, colon, cervix, prostate, and epithelial-type mesothelioma (13, 17, 22, 44, 47–50, 52, 58). HAI-1, a type II transmembrane protein, contains two Kunitz, aprotinin-like serine protease inhibitory domains, and an LDL receptor class A domain. This inhibitor was initially identified from gastric cancer cells as a potent inhibitor of HGF activator, a liver-derived, blood-borne serine protease (38, 51). Subsequently, HAI-1 was identified and isolated from human milk and breast cancer cells in complexes with matriptase (29–31). Recently, HAI-1B, a splice variant of HAI-1, a type I transmembrane protein, contains two Kunitz, aprotinin-like serine protease inhibitory domains, and an LDL receptor class A domain. This inhibitor was initially identified from gastric cancer cells as a potent inhibitor of HGF activator, a liver-derived, blood-borne serine protease (38, 51). Subsequently, HAI-1 was identified and isolated from human milk and breast cancer cells in complexes with matriptase (29–31). Recently, HAI-1B, a splice variant of HAI-1, was shown to inhibit prostatin, a glycosylphosphatidylinositol-anchored serine protease (10). Besides its role in the inhibition of matriptase, HAI-1 is paradoxically required for matriptase activation (46) and is involved in the expression and trafficking of matriptase (43).

The role of matriptase in tumor onset and progression goes beyond its deregulated expression and imbalance to HAI-1 in human cancers. For example, the stability and proteolytic activity of matriptase can be enhanced by modification of its glycosylation status by β1,6-N-acetylgalactosaminyltransferase V, a prometastatic enzyme (15, 16). Its deregulation in breast cancer cells also occurs at the level of activation. In mammary epithelial cells, matriptase activation depends on the presence of
of sphingosine 1-phosphate (S1P), a blood-borne lysosphospholipid (2, 3); breast cancer cells, however, constitutively activate matriptase regardless of the presence of S1P (4). The balance of matriptase and HAI-1 levels also affects the activation of matriptase, and reduced expression of HAI-1 by small interfering RNA results in spontaneous activation of matriptase and enhanced S1P-induced matriptase activation in human mammary epithelial cells (43). Recently, both matriptase and HAI-1 were identified by quantitative proteomic analysis as cellular proteins shed into culture media in response to androgen exposure in LNCaP prostate cancer cells, along with the canonical androgen responsive protease, prostate-specific antigen (PSA) (35). Because ectodomain shedding of matriptase and HAI-1 closely follow the activation of matriptase (2), androgen exposure might also cause activation of matriptase in prostate cancer cells. Therefore, we proposed that matriptase activation might be regulated by steroid sex hormones and so we initiated the current study to investigate matriptase activation in response to steroid sex hormones, in both prostate and breast cancer cells.

MATERIALS AND METHODS

Chemicals and reagents. Formaldehyde solution was purchased from EM Science (Bibbstown, NJ). 5α-Dihydrotestosterone (DHT), 17β-estradiol (E2), and 4-hydroxytamoxifen (OHT) were obtained from Sigma (St. Louis, MO). Actinomycin D and cycloheximide were obtained from Bioworld (Plymouth Meeting, PA). Bicalutamide was obtained from Toronto Research Chemicals (North York, ON, Canada). All other chemical reagents were obtained from Sigma unless otherwise specified.

Cell culture conditions. Human cancer cells were obtained from the Tissue Culture Shared Resource of the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center. The breast cancer cells (T-47D, MCF-7, and MDA MB-468) were maintained in culture by growth in Iscove’s minimal essential media (IMEM; Invitrogen, Rockville, MD) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified chamber at 37°C and 5% CO2. LNCaP, PC-3, and DU145 human prostate cancer cells were routinely maintained in RPMI 1640 supplemented with 5% FBS, 1% glutamine, and 0.5% gentamicin in a humidified chamber at 37°C and 5% CO2 (41).

To investigate the effects of estrogen on breast cancer cells, T-47D (2 × 10^3/cm²), MCF-7 (5 × 10^3/cm²), and MDA MB-468 (3 × 10^3/cm²) were cultured for 2 days, washed once with phosphate-buffered saline (PBS), and subjected to serum and hormone starvation in steroid-reduced IMEM [10 mM HEPES-buffered (pH 7.2) phenol red-free IMEM, containing 100 mM penicillin and 100 μg/ml streptomycin], containing 5% charcoal/dextran-treated FBS (Hyclone, Logan, UT), and 1 μM OHT for a day, followed by steroid-reduced IMEM containing 1% charcoal/dextran-treated FBS and 0.2 μM OHT for an additional day. The cultures were then washed twice with PBS and treated with OHT or E2 in a steroid-reduced IMEM for 24 h. To investigate androgen effects on prostate cancer cells, LNCaP (3 × 10^3/cm²), PC-3 (5 × 10^3/cm²) and DU145 (5 × 10^3/cm²) were cultured 2 days, washed once with PBS, and then subjected to serum and hormone starvation in a steroid-reduced RPMI 1640 [10 mM HEPES-buffered (pH 7.2), phenol red-free RPMI 1640, containing 1% glutamine and 0.5% gentamicin], containing 5% charcoal/dextran-treated FBS for a day, followed by culture in steroid-reduced RPMI containing 1% charcoal/dextran-treated FBS for an additional day. The cultures were then washed once with PBS and treated with DHT in a steroid-reduced RPMI for the indicated period.

Cell viability assay. Because cycloheximide and actinomycin D are known to be somewhat toxic, the viability of LNCaP cells after cycloheximide or actinomycin D treatment was monitored by Trypan blue exclusion. Those treated cells were trypsinized, collected, and stained with Trypan blue solution according to the commercial protocol. Under a microscope, live and dead cells were counted in a hemocytometer. The majority of cells (from 90% to 80%) were viable after these treatments.

Monoclonal antibodies. Human matriptase protein was detected using either the M32 monoclonal antibody, which recognizes the third LDL receptor class A domain of matriptase of both the latent (one chain) and activated (two chain) forms of the protease, or using the M69 monoclonal antibody, which recognizes an epitope present only in the activated (two chain) form of the enzyme (2, 3). Human HAI-1 was detected using the HAI-1 specific monoclonal antibody M19 (29). Characterization of mAbs used in the current study, including M69, M32, and M19 is summarized in Table 1.

Western blot analysis. Protein for Western blot analysis was prepared by lysis of cells in 1% Triton X-100 in PBS, after the cells were washed two times in PBS. Insoluble debris was removed by centrifugation, and the protein concentration was determined using the BCA protein assay reagents (Pierce, Rockford, IL) according to the manufacturer’s protocol. Lysates were diluted in a 5× sample buffer. The sample buffer did not contain a reducing agent, and samples were not boiled before SDS-PAGE administration because reducing agents destroy the epitopes recognized by the monoclonal antibodies, and boiling disrupts matriptase/HAI-1 complexes. Proteins were resolved by 7.5% SDS-PAGE, transferred to Protran nitrocellulose membranes (Schleicher and Schuell, Keene, NH), and probed with monoclonal antibodies M32, M69, and M19, and with an antibody that recognizes human PSA (OriGene, San Diego, CA). The binding of the primary antibody was followed by recognition with a goat anti-mouse-HRP conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected using the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA).

RNA preparation and analysis. Cells for RNA studies were cultured and treated with steroids as described. Total RNA was prepared using Tri Reagent (Sigma), following the manufacturer’s instructions, and RNA quality and concentration was determined by absorbance using Tri Reagent (Sigma), following the manufacturer’s instructions, and RNA quality and concentration was determined by absorbance measurements and electrophoretic analysis on denaturing agarose gels. Matriptase and PSA mRNA levels were measured by real-time quantitative PCR using primers designed for qRT-PCR.

Table 1. Summary of various forms of matriptase and HAI-1 and mAbs

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Matriptase forms

HAI-1 forms

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HAI-1, hepatocyte growth factor activator inhibitor-1.
RT-PCR using standard methods. Briefly, aliquots of total RNA were treated with RQ1 RNase-free DNase (Promega) to remove any contaminating genomic DNA and then used to generate cDNA using the avian myoblastosis virus-reverse transcriptase system (Promega) per the manufacturer’s instructions. The resultant cDNA was subjected to real-time singleplex PCR using Assays-on-Demand gene expression products [20× mix of unlabeled PCR primers and hexachloro-6-carboxy-fluorescence (FAM) or VIC dye-labeled TaqMan MGB probe] specific for matriptase, PSA, or GAPDH (AB Applied Biosystems) according to manufacturer’s instructions. The relative standard curve method was used to quantify the expression levels of each gene. GAPDH was used as the endogenous reference to normalize samples and results are expressed relative to the level in control cells. The means ± SD of triplicate determinations are presented.

RESULTS

Androgen induces matriptase activation and ectodomain shedding in LNCaP prostate cancer cells. To test the hypothesis that steroid sex hormones stimulate matriptase activation and subsequent ectodomain shedding of this cognate, protease-inhibitor pair, we examined the effects of steroid sex hormones on the levels of activated matriptase, total matriptase (mainly in its latent form), and HAI-1 in androgen-sensitive human prostate cancer LNCaP cells, androgen-insensitive PC-3 and DU145 cells, as well as estrogen-sensitive breast cancer cells MCF-7 and T-47D cells and estrogen-insensitive MDA MB-468 cells. Activation of matriptase was expressed as the combined total of activated matriptase in the cell lysate plus that detected in the conditioned media, using mAb M69. This mAb specifically recognizes the two-chain activated form of matriptase, but not the single-chain, latent enzyme (2). HAI-1 is known to be paradoxically required for matriptase activation (46). Thus, due to the presence of HAI-1 together with matriptase in activation foci (23), the active matriptase quickly binds to HAI-1 and is detected in cell lysates as complexes with HAI-1 either at 120-kDa complex (which contains 70-kDa matriptase and full-length 55-kDa HAI-1) or at 85-kDa complex (which contains full-length HAI-1 and probably the serine protease domain of matriptase) (Table 1) (23). In the conditioned media, matriptase-HAI-1 complexes are detected either at 110 kDa (which contains 70-kDa matriptase and 50-kDa HAI-1 fragment) or at 95 kDa (which contains 70-kDa matriptase and 40-kDa HAI-1 fragment) (see Table 1) (29). Because active matriptase is inhibited and bound with HAI-1 after its activation, determination of matriptase activity levels by using synthetic substrates or gelatin zymography is not feasible (2). Furthermore, active matriptase, but not latent matriptase, can bind to HAI-1 to form matriptase-HAI-1 complexes, which exhibit different migration rates on SDS-PAGE, compared with those of latent and noncomplexed active matriptase. Because of the combination of the specificity of mAb M69 and the shift of migration rates on SDS gel, the status of matriptase activation can be accurately and conveniently determined by Western blot analysis using mAb M69.

Low levels of activated matriptase were detected in all three hormone-starved prostate cancer cell lines, either in cell lysates or in conditioned media. In contrast to PC-3 and DU145 cells, which showed no obvious response to androgen with regard to matriptase activation (data not shown), in the androgen-sensitive LNCaP cells, DHT induced matriptase activation in a dose-dependent manner (Fig. 1A; M69). The levels of activated matriptase, both in the cell lysate and in the conditioned media, were significantly increased by the treatment with 10−9 M DHT, compared with those of untreated cells (Fig. 1A). While there was no obvious difference in the cellular levels of activated matriptase, comparing 10−9 M DHT to 10−8 M DHT (Fig. 1A, M69 cell lysate), treatment with 10−8 M DHT resulted in the presence of much more activated matriptase in the conditioned media (Fig. 1A, M69 medium). These data suggest that matriptase activation in LNCaP prostate cancer cells is regulated by androgen, and that the majority of activated matriptase is shed to the extracellular milieu, resulting in
relatively constant levels of activated matriptase present in the cells themselves.

In conjunction with DHT-induced activation and shedding of activated matriptase, androgen also caused shedding of latent matriptase into conditioned media in a dose-dependent manner (Fig. 1A, M32 medium). As a result of this induced shedding of the protease, cellular levels of latent matriptase decreased in response to DHT treatment in a dose-dependent manner (Fig. 1A, M32 cell lysate). Levels of latent matriptase were determined using mAb M32 (Table 1), which recognizes the third LDL receptor class A domain of matriptase and interacts with both latent and activated matriptase (14). However, because activated matriptase always binds to available HAI-1, and because activated matriptase always represents a small proportion of total cellular matriptase, the 70-kDa band recognized by mAb M32 is used for determining the level of latent matriptase, and represents the majority of the protease in cell lysate. In light of the profound effects of androgen treatment on the degree of matriptase activation and the cellular levels of the protein, we determined whether androgen treatment was altering matriptase expression at the RNA level. RNA was prepared from cells treated with androgen exactly as in the protein studies and mRNA for matriptase and the canonical androgen-responsive gene PSA were assayed by real-time RT-PCR. As expected, PSA mRNA levels were significantly induced by androgen treatment, whereas matriptase message levels were essentially unchanged (Fig. 1B).

DHT also caused shedding of HAI-1 in a dose-dependent manner (Fig. 1, M19 medium), again at the cost of cellular levels of the inhibitor (Fig. 1A, M19 cell lysate). We detected HAI-1 using mAb M19, which recognizes full-length inhibitor at 55-kDa in cell lysates, and 50- and 40-kDa shed fragments in the conditioned media (Table 1). This mAb also recognizes matriptase-HAI-1 complexes, either in cell lysates at 120 and/or 85 kDa, or in conditioned media at 95 and/or 110 kDa (Table 1) (23, 29). Because the ratio of 120- and 85-kDa complexes, relative to the free form of HAI-1, is sometimes very low, both complexes may not be seen in cell lysate using this mAb.

After hormone starvation, in contrast to prostate cancer cells, breast cancer cells, including T-47D, MCF-7, and MDA MB-468 cells, maintained high levels of activated matriptase, both in cell lysates and conditioned media. Treatment with E2 (0.1 to 10 nM) or 1 μM OHT resulted in no obvious change in the levels of activated matriptase nor the total matriptase and HAI-1 (data not shown). While some breast cancer cells (T-47D, MCF-7, and MDA MB-453) also express androgen receptor (8), there was no obvious change in the levels of matriptase activation in these breast cancer cells after exposure of DHT (data not shown). This may be due to the high levels of activated matriptase in hormone-starved breast cancer cells. Interestingly, E2 (10 nM) can induce matriptase activation and shedding in LNCaP prostate cancer cells (data not shown). This is probably because these cells express a mutated form of the androgen receptor that is known to bind estradiol (60).

Kinetics of DHT effects on matriptase activation and ectodomain shedding of matriptase and HAI-1 in LNCaP prostate cancer cells. We determined the time course of the DHT effect on matriptase activation, and the temporal relationship of matriptase activation and ectodomain shedding of the protease and the inhibitor (Fig. 2). Activated matriptase appeared in LNCaP cell lysates 6 h after DHT treatment, accumulated to the highest level at 18 h, and decreased at 24 h (Fig. 2; M69 cell lysate). Consistent with the appearance of activated matriptase in cell lysates 6 h after DHT treatment, activated matriptase also began to appear in culture media, whereas at very low levels, 6 h after DHT treatment (Fig. 2, M69 medium). Continuous accumulation of activated matriptase was observed, reaching very high levels 24 h after DHT treatment (Fig. 2, M69 medium). The cellular levels of 70-kDa, latent matriptase, were similar in control and DHT-treated cells, up to 3 h after DHT treatment (Fig. 2, M32 Cell lysate). However, after 6 h of DHT treatment, when the activation began to occur, the cellular levels of latent matriptase began to decrease, and...
were maintained at low levels for up to 24 h after treatment (Fig. 2, M32 cell lysate). This long-term decrease in matriptase levels was consistent with the conversion of latent-to-activated matriptase and shedding of the protease to culture media. Accompanying the decrease in cellular levels of matriptase during its activation, 70-kDa latent matriptase accumulated to very high levels 12 h after DHT treatment (Fig. 2, M32 medium). Similar to the protease, cellular levels of HAI-1 were stable before matriptase activation, and decreased during activation of matriptase (Fig. 2, M19 cell lysate). Shedding of HAI-1 appeared to be more complex than matriptase. After stimulation of DHT-starved cells, shedding of the 50-kDa HAI-1 fragment was detected for only 3 h, and the 40-kDa fragment was detected after 12 h (Fig. 2, M19 medium). DHT treatment did not increase the shedding of the 50-kDa HAI-1 fragment, but significantly boosted the levels of the 40-kDa HAI-1 fragment in culture media. The shedding of HAI-1 in its 95-kDa complex, which contains activated matriptase and 40-kDa HAI-1 fragment, totally depended on the presence of DHT and followed the same pattern as activated matriptase (Fig. 2, M19 medium). While DHT-induced shedding of HAI-1 did cause a decrease in its cellular levels, the constitutive shedding of the 50-kDa HAI-1 fragment in the absence of DHT did not result in reduced cellular levels of HAI-1 (Fig. 2, M19 cell lysate). This could represent a balance between biosynthesis and shedding in the absence of androgen. Treatment of cells with androgen apparently potentiated shedding but did not increase the biosynthesis of HAI-1 enough to maintain cellular levels of the inhibitor. These data also suggest that there may be two different proteases responsible for the shedding of HAI-1: one protease that converts the 55-kDa, membrane-bound, full-length HAI-1 to a 50-kDa fragment, and another to convert the 55-kDa HAI-1 to the 40-kDa fragment. The former could occur constitutively, the latter being enhanced by androgen exposure and/or by activation of matriptase.

We also examined the kinetics of the expression and secretion of PSA, in response to DHT treatment (Fig. 2, PSA). Increased expression of PSA in LNCaP cells was observed at 6 h after DHT treatment, with cellular levels reaching a plateau in the cell lysates at 18 h (Fig. 2, PSA cell lysate). Secretion of PSA began to be detected at low levels at 12 h after DHT treatment, accumulating to very high levels by 24 h (Fig. 2, PSA medium). A balance between biosynthesis and secretion of PSA seemed to be established at 18 h after DHT treatment. Although there were similar time course kinetics for DHT-induced release of matriptase and PSA, the shedding of matriptase is closely coupled to the activation of the protease, rather than its protein expression. The secretion of PSA appeared to be more closely coupled to its expression.

**Androgen receptor is involved in DHT-induced activation and shedding of matriptase.** We examined the molecular mechanisms involved in androgen-induced matriptase activation and its subsequent shedding. Cellular responses to androgen are mainly mediated via the activation of the intracellular androgen receptor (AR) (21, 25, 63) or the putative cell surface receptor, likely to be a G protein-coupled receptor (5). Thus we investigated whether AR is involved in DHT-induced matriptase activation and shedding by using the AR antagonist, bicalutamide (6, 37), and by using albumin-conjugated testosterone, which cannot penetrate through the plasma membrane to activate the intracellular AR (27). While bicalutamide did not alter cellular levels of DHT-induced activated matriptase (Fig. 3A, M69 cell lysate), the AR antagonist significantly reduced the levels of DHT-induced activated matriptase in the conditioned media (Fig. 3A, M69 medium). Because the shed, activated matriptase represents >90% of the total activated matriptase, these data clearly suggest that bicalutamide significantly inhibits DHT-induced matriptase activation and that AR is involved in mediating the effect of DHT on matriptase activation. Accordingly, the cellular level of latent matriptase was much higher (Fig. 3A, M32 cell lysate) and the shed latent matriptase was lower (Fig. 3A, M32 medium) in bicalutamide- pretreated cells, compared with cells treated with DHT alone. HAI-1

**![Image](http://ajpcell.physiology.org/)**

Fig. 3. Androgen induced matriptase activation in LNCaP cells via the androgen receptor. A: hormonally starved LNCaP cells were untreated or pretreated with 10 μM bicalutamide for 30 min, followed by the addition of DHT for another 24 h in serum and phenol red-free, 10 mM HEPES-buffered (pH 7.2) RPMI 1640. The conditioned media and cell lysates were collected, and equal amounts of proteins from cell lysates and equal volumes of concentrated cultured media were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Activated matriptase, total matriptase, HAI-1, and PSA were detected by immunoblotting with mAbs M69, M32, M19, and anti-PSA Ab, respectively. B: hormonally starved LNCaP cells were treated with 10−8 M DHT or BSA-testosterone (BSA-T) at 100 nM in serum and phenol red-free medium for 24 h. The levels of activated matriptase in cell lysate and conditioned media were determined by immunoblot using mAb M69.
shared a similar trend but less obvious trend (Fig. 3A, M19). The inhibition of DHT-induced PSA expression by bicalutamide was included as an experimental control (Fig. 3A, PSA).

In a manner consistent with the selective involvement of intracellular AR in activation of matriptase, BSA-testosterone at 100 nM failed to induce matriptase activation and shedding (Fig. 3B).

The involvement of AR in matriptase activation and shedding suggests that transcription of AR-targeted genes may play a role in matriptase activation and shedding. This hypothesis was further confirmed by the inhibition of DHT-induced matriptase activation and shedding by cycloheximide, a protein synthesis inhibitor (55, 61), and actinomycin D, a widely used transcription inhibitor (7, 36). In Fig. 4A, we show the effects of DHT on the activation and shedding of matriptase in the presence of cycloheximide or actinomycin D. While activated matriptase was still detected in lysates of cycloheximide-pretreated cells, much lower levels of shed activated matriptase, shed latent matriptase, and shed HAI-1 were detected (Fig. 4A, medium). Consistent with this, the cellular levels of latent matriptase and HAI-1 were higher in cycloheximide-pretreated cells than in those treated with DHT alone (Fig. 4A, cell lysate). The inhibition by actinomycin D of DHT-induced matriptase activation and shedding showed similar trends to what was seen with cycloheximide, but actinomycin D seemed to produce a much more potent inhibition. Activated matriptase was not detected in cell lysates and conditioned media from actinomycin D-pretreated cells (Fig. 4A, M69). Latent matriptase was detected in actinomycin D-pretreated cells at similar levels to those in hormone-starved cells, and there was no detectable, latent matriptase in conditioned media (Fig. 4A, M32). Cellular levels of HAI-1 in actinomycin D-pretreated cells were similar to those of hormone-starved cells (Fig. 4A, M19 cell lysate). The shedding of the 95-kDa matriptase-HAI-1 complex and the 40-kDa HAI-1 fragment, but not the 50-kDa HAI-1 fragment, was significantly inhibited by actinomycin D (Fig. 4A, M19 medium). This was consistent with the fact that shedding of the former two forms depended on DHT, but shedding of the 50-kDa HAI-1 fragment is constitutive (Fig. 2). However, the constitutive shedding of the 50-kDa HAI-1 fragment was inhibited by cycloheximide (Fig. 4A, M19 medium). This could result from the decreased synthesis of HAI-1 protein. Both cycloheximide and actinomycin D inhibited PSA expression and secretion, a known AR-dependent event (Fig. 4A, PSA). We also examined how the effect of combinations of DHT with cycloheximide or actinomycin D affected the mRNA expression of matriptase and PSA (Fig. 4B). The protein synthesis inhibitor, cycloheximide, did significantly reduce PSA message levels but did not alter levels of matriptase mRNA. In contrast, the transcription inhibitor, actinomycin D, significantly reduced the mRNA levels of matriptase. The involvements of AR activation, RNA transcription, and protein synthesis in DHT-induced matriptase activation are consistent with the time period required for DHT to induce matriptase activation (Fig. 2). These events require at least a few hours for the AR pathways to respond to DHT treatment, due to the involvement of RNA transcription and protein biosynthesis. On the other hand, one might expect that the effect would be much more rapid if mediated by a G protein-coupled receptor involving signaling that did not require alterations in protein synthesis.

Fig. 4. Androgen-induced matriptase activation in LNCaP cells is involved in RNA transcription and protein synthesis. Hormonally starved LNCaP cells were untreated or pretreated with cycloheximide (CHX) or actinomycin D (Act D) for 30 min, followed by the addition of DHT for another 24 h in serum and phenol red-free, 10 mM HEPES-buffered (pH 7.2) RPMI 1640. A: equal amounts of proteins from cell lysates and equal volumes of concentrated cultured media were subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Activated matriptase, total matriptase, HAI-1, and PSA were detected by immunoblotting with mAbs M69, M32, M19, and anti-PSA Ab, respectively. B: total RNA was prepared and assayed for matriptase, PSA, and GAPDH mRNA levels. Matriptase and PSA levels were normalized for GAPDH level and then expressed relative to the levels in control cells. The means ± SD of triplicate determinations are presented.

AJP-Cell Physiol • VOL 291 • JULY 2006 • www.ajpcell.org
Androgen-induced shedding of matriptase and HAI-1 involves proteolytic cleavages. Because the sizes of shed HAI-1 are either 50 or 40 kDa, smaller than the 55-kDa, membrane-associated species (Figs. 1 and 2), the shedding of HAI-1 is apparently carried out by proteolytic cleavages that release the HAI-1 extracellular fragments from its transmembrane anchor on cell surfaces. While matriptase undergoes NH2-terminal processing by proteolytic cleavage between Gly-149 and Ser-150 within its sperm protein-enterokinase-agrin module, the bulk of its extracellular domains are still bound to its transmembrane domain via an unknown mechanism (9, 46). The shedding of matriptase extracellular domains could occur simply by dissociation of its extracellular fragment from its transmembrane domain, and the size of matriptase from the conditioned media is expected to be the same of that from cell lysates. However, when the apparent sizes of matriptase from cell lysate and conditioned media were compared, the medium-derived matriptase exhibited a faster migration rate compared with that of matriptase in cell lysates on SDS gel (Fig. 5). These results suggest that the matriptase present in the medium is not identical to and with a smaller size than membrane-bound matriptase. Therefore the simple dissociation of the extracellular domains of matriptase from its transmembrane domain is not the mechanism for matriptase shedding, and proteolytic cleavage of matriptase is likely to be involved in the shedding of the protease. In a previous study (2), we identified that the amino termini of matriptase proteins isolated from the milk-derived, 95-kDa matriptase-HAI-1 complex to be Ser-189 and Thr-204, instead of Ser-150. These results suggest that the matriptase present in the medium is not identical to and with a smaller size than membrane-bound matriptase. Therefore the simple dissociation of the extracellular domains of matriptase from its transmembrane domain is not the mechanism for matriptase shedding, and proteolytic cleavage of matriptase is likely to be involved in the shedding of the protease. In a previous study (2), we identified that the amino termini of matriptase proteins isolated from the milk-derived, 95-kDa matriptase-HAI-1 complex to be Ser-189 or Thr-204, instead of Ser-150. These results suggest that cleavages between Lys-188 and Ser-189 or between Lys-203 and Thr-204 must occur for matriptase shedding, leading to the presence of smaller forms of matriptase in extracellular milieu. Taken together, these data suggest that shedding of matriptase-HAI-1 complexes could require simultaneous and well-coordinated proteolytic cleavages of this cognate pair of proteolytic enzyme and inhibitor.

**DISCUSSION**

In the present study we describe a series of androgen-induced events which regulate cell surface proteolysis of prostate cancer cells. Exposure of LNCaP prostate cancer cells to androgen first activates AR, leading to RNA transcription and biosynthesis of protein(s), which play critical roles in the activation of matriptase. This androgen-induced activation of matriptase was rapidly followed by the inhibition of active matriptase by binding to its cognate inhibitor HAI-1 and then clearance of the matriptase-HAI-1 complex from cell surfaces. As described in our previous studies (2), activation of matriptase requires a proteolytic cleavage at its activation motif to convert a single-chain zymogen to an active, disulfide-linked, two-chain protease, a process conserved among many serine proteases. While the activational cleavage of serine proteases is mainly executed by other active proteases, the activation of matriptase is likely to be carried out by its own, intrinsic proteolytic activity of zymogen via protein-protein interactions among matriptase zymogens, HAI-1, and other as-yet unidentified molecules (43, 46). During the process of matriptase activation, these required components of the matriptase activation machinery are observed to translocate to and accumulate at activation foci, specialized subcellular locations where activation of matriptase occurs (14, 23). Androgen treatment may induce protein expression of these yet unidentified components required for matriptase activation. Alternatively, the AR-targeted genes could indirectly facilitate matriptase activation, by creating the proper microenvironment for matriptase activation, or by facilitating their translocation to the activation foci. Identification of these AR-targeted genes in the future will likely be the key to understanding how androgen stimulates activation of matriptase.

After matriptase activation, inhibition of active matriptase appeared to occur immediately via the binding of active matriptase with its cognate inhibitor HAI-1, since all activated matriptase, both in the cell and in conditioned media, was detected in HAI-1 complexes. This rapid and efficient inhibition of matriptase by HAI-1 could result from the required participation of HAI-1 in matriptase activation (46) and the presence of HAI-1 with matriptase in activation foci (23). This mode of inhibition of matriptase by HAI-1 in prostate cancer cells seems to be identical to that observed in mammary epithelial cells, in which activation of matriptase can be induced by S1P and suramin. The shedding of matriptase-HAI-1 complexes occurred as early as 6 h after the cellular treatment with DHT and temporally correlated with the onset of matriptase activation (Fig. 2). Because proteolytic cleavages of both matriptase and HAI-1 are required for the removal of the matriptase-HAI-1 complex from cell surfaces, a well-coordinated mechanism, probably involving other proteases, could be turned on right after activation and inhibition of matriptase. This cellular clearance of activated matriptase could provide a final mechanism to remove active matriptase, a potentially biohazardous molecule.

Whereas all of these well-coordinated events are triggered by androgen, the questions of how and when matriptase acts on its substrates are a major missing link in our understanding of matriptase functionality and regulation. Because the half-life of active matriptase is likely to be very short, it is plausible that the physiological substrates of matriptase (as yet largely unknown) could also be present where matriptase activation occurs, and conceivably, these substrates may even participate in matriptase activation. Because of the close association between matriptase activation and its shedding with HAI-1, a process which also requires proteolytic cleavage at both HAI-1 and matriptase, we propose that some of the matriptase substrates may be other membrane-bound proteases and could be involved in the shedding of matriptase and HAI-1. Thus
matriptase may activate the protease(s) responsible for its own shedding; removal of activated matriptase from cell surfaces may be closely coupled to the activation of matriptase.

Two matriptase-HAI-1 complexes of 110 and 95 kDa were detected in the conditioned media of breast cancer cells. Both the 110- and 95-kDa complexes contain 70-kDa matriptase. Their size differences reside in the sizes of HAI-1 fragments. The 110-kDa complex contains the 50-kDa HAI-1 fragment and the 95-kDa complex contains the 40-kDa HAI-1 fragment. How are these two HAI-1 fragments generated and are they the products of one protease or two different proteases? This study of LNCaP cells provides some clues to this question. Despite constitutive shedding of the 50-kDa HAI-1 fragment, LNCaP prostate cancer cells shed the 95-kDa complex and the 40-kDa HAI-1 fragment, but not the 110-kDa complex in response to androgen treatment. These data suggest that LNCaP cells may express two different proteases responsible for the shedding of HAI-1: one protease being constitutively active and responsible for the shedding of the 50-kDa HAI-1 fragment and another protease that becomes active only in response to androgen treatment and cleaves HAI-1 at a different site resulting in the shedding of the 40-kDa HAI-1 fragment and the 95-kDa matriptase-HAI1-complex.

The impact of matriptase activation on LNCaP prostate cancer cell biology may lie in the substrates, which are directly or indirectly activated by matriptase. Previously, three substrates of matriptase were identified in an in vitro setting, including urokinase-type plasminogen activator, HGF/scatter factor, and protease-activated receptor-2 (PAR-2) (20, 24, 53, 56). To our knowledge, neither the urokinase-type plasminogen activator nor HGF is expressed by LNCaP cells. Therefore, the role of matriptase in cancer invasion and metastasis via urokinase and HGF may require interaction with stromal cells in vivo in some prostate cancers. Interestingly, the G protein-coupled receptor PAR-2 is expressed by LNCaP cells and participates in the activation of the small GTPase RhoA and subsequent actin filament rearrangement (12). It is of interest for future studies to determine whether DHT can cause activation of PAR-2 via the activation of matriptase.

Besides matriptase and PSA, several proteases, including human gland kallikrein (62), a protease with high homology with enameled matrix serine proteinase 1 (39), protease/KLK-L1 (64), TMPRSS2 (1, 28), and MMP-2 (26), are regulated by androgen in prostate cells. Matriptase is unique among these androgen-regulated proteases in that its regulation by androgen occurs mainly at the level of activation. While androgen can enhance the activation of these other androgen-regulated proteases (1), the increased protease activation mainly results from its increased expression. The tight coupling of matriptase shedding with its activation also distinguishes matriptase from the other androgen-regulated proteases. While both matriptase and PSA are released into the extracellular milieu in response to androgen exposure, the mechanisms by which androgens induce their release are very different. In contrast to PSA, whose expression and secretion were induced by androgen, prostate cancer cells constitutively express matriptase and HAI-1. Androgen clearly works to selectively induce ectodomain shedding of matriptase and HAI-1, as a consequence of androgen-induced activation of matriptase. While both matriptase and HAI-1 were shed either in free form or complexed forms in cultured cells, we have previously detected only the complexed form of the enzyme in human milk (29). These observations suggest that under physiological conditions such as lactation, ectodomain shedding occurs only for the matriptase-HAI-1 complex, and not for latent matriptase and free HAI-1. It remains to be determined whether shedding of latent matriptase and free HAI-1 fragments are associated with cell culture conditions or with the malignant progression.

GRANTS

This study was supported by National Institutes of Health Grants R01-CA-104944 (to C.-Y. Lin) and R01-CA-096851 (to R. B. Dickson). C.-Y. Lin and M.-S. Lee received salary support by Department of Defense Grants DAMD17-01-1-0252 and DAMD17-01-1-0271, respectively. This work was supported in part by the Lombardi Comprehensive Cancer Center Microscopy and Imaging and Tissue Culture Shared Resource, National Cancer Institute Grant 4P30-CA-51008, and a grant from Dendreon Corp.

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