Pro-urokinase-type Plasminogen Activator Is a Substrate for Hepsin*

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Hepsin, a type II transmembrane serine protease, is strongly up-regulated in prostate cancer. Hepsin overexpression in a mouse prostate cancer model resulted in tumor progression and metastasis, associated with basement membrane disorganization. We investigated whether hepsin enzymatic activity was linked to the basement membrane defects by examining its ability to initiate the plasminogen/plasmin proteolytic pathway. Because plasminogen is not processed by hepsin, we investigated the upstream activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator. Enzymatic assays with a recombinant soluble form of hepsin demonstrated that hepsin did not cleave pro-tissue-type plasminogen activator but efficiently converted pro-uPA into high molecular weight uPA by cleavage at the Lys158-Ile159 (P1-P1') peptide bond. uPA generated by hepsin displayed enzymatic activity toward small synthetic and macromolecular substrates indistinguishable from uPA produced by plasmin. The catalytic efficiency of pro-uPA activation by hepsin (kcat/Km = 4.8 × 105 M−1 s−1) was similar to that of plasmin, which is considered the most potent pro-uPA activator and was about 6-fold higher than that of matriptase. Conversion of pro-uPA was also demonstrated with cell surface-expressed full-length hepsin. A stable hepsin-overexpressing LnCaP cell line converted pro-uPA into high molecular weight uPA at a rate of 6.6 ± 1.9 nM uPA h−1, which was about 3-fold higher than LnCaP cells expressing lower hepsin levels on their surface. In conclusion, the ability of hepsin to efficiently activate pro-uPA suggests that it may initiate plasin-mediated proteolytic pathways at the tumor/stroma interface that lead to basement membrane disruption and tumor progression.

Hepsin is a type II transmembrane serine protease expressed on the surface of epithelial cells. The 417-amino acid protein is composed of a short N-terminal cytoplasmic domain, a transmembrane domain, and a single scavenger receptor cysteine-rich domain that packs tightly against the C-terminal protease domain (1). The physiologic function of hepsin remains unknown. Despite its expression in the very early stages of embryogenesis (2), hepsin-deficient mice were viable and developed normally (3, 4). The studies further showed that hepsin was not essential for liver regeneration and for coagulation-related physiological functions (3, 4). However, hepsin has been implicated in ovarian cancer (5) and prostate cancer (6–11), where several gene expression studies have identified it as one of the most highly induced genes. Hepsin RNA levels were found to be low in normal prostate and benign hyperplasia but strongly increased in prostate carcinoma, particularly in advanced stages (8–10). Hepsin protein staining with a monoclonal anti-hepsin antibody showed that hepsin expression was highest at sites of bone metastasis and in late stage primary tumors (12), which is consistent with the finding that increased hepsin RNA levels correlated with higher Gleason grades and tumor progression (7–10, 13). In contrast, using a different antibody Dhanasekaran et al. (6) found the strongest hepsin expression in high grade prostate intraneplastic lesions and lower expression in primary carcinoma and in metastatic lesions. These studies raised the question of whether hepsin is involved in prostate cancer. In vitro studies did not provide clear answers, and depending on the experimental conditions used, hepsin was found to promote, inhibit, or not affect tumor cell growth (12, 14, 15).

Evidence for a role of hepsin in prostate cancer came from a recent study by Klezovitch et al. (16) demonstrating that in a mouse model of non-metastasizing prostate cancer, overexpression of hepsin led to primary tumor progression and metastasis. Intriguingly, hepsin overexpression was associated with basement membrane disruption (16) pointing toward the possibility that hepsin activity is somehow linked to the degradation of basement membrane components. In vitro, hepsin was able to convert the latent growth factor pro-hepatocyte growth factor (pro-HGF)2 into its active two-chain form (HGF), which induced Met receptor signaling (17, 18). Because the HGF/Met pathway has been implicated in invasive tumor growth and metastasis, it is possible that overexpression of hepsin activates the HGF/Met axis in prostate cancer. Hepsin was also shown to cleave other substrates in vitro, mainly coagulation-related proteins (17, 19). However, their role in tumorigenesis is not known.

In view of the basement membrane defects that were associated with hepsin overexpression in the mouse prostate, we hypothesized that hepsin might activate protease zymogens that are directly linked to basement membrane degradation. It was known from previous studies that hepsin does not activate

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The abbreviations used are: HGF, hepatocyte growth factor; HGFA, HGF activator; KD1, Kunitz domain-1 derived from HAI-1; pNA, para-nitroanilide; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; pro-uPA, zymogen form of uPA; pro-tPA, zymogen form of tPA; pro-HGF, single chain form of HGF; TAMRA, 6-carboxytetramethylrhodamine; FAM, 6-carboxyfluorescein; PBS, phosphate-buffered saline.
plasminogen (17, 18), and therefore, we considered the upstream activators of plasminogen, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) as possible candidates. The results of this study show that soluble hepsin is a potent activator of pro-uPA with a catalytic efficiency comparable with that of plasmin. Pro-uPA conversion was also measured by hepsin-expressing LnCaP prostate cancer cells, demonstrating that cellular full-length hepsin recapitulates the function of soluble hepsin. The implications of these findings with respect to the function of hepsin in prostate cancer progression are discussed.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Lys-plasmin and Lys-plasminogen were from Haematologics Technologies Inc. (Essex Junction, VT). Pro-tPA was from Biodesign International (Saco, ME), uPA (high molecular weight form) was from American Diagnostica (Greenwich, CT), pro-uPA was from Cortex Biochem (San Leandro, CA), and PAI-1 was from Molecular Innovations (Southfield, MI). T.in.Pro cells were from Expression System LLC (Woodland, CA). Nickel-nitrilotriacetic acid resin was from Qiagen Inc. (Chatsworth, CA), and PAI-1 was from Molecular Innovations (Southfield, MI). The chromogenic substrates S2444, S2765, and S2366 were from Diapharma (Westchester, OH). T.in.Pro cells were from Expression System LLC (Woodland, CA). Nickel-nitrilotriacetic acid resin was from Qiagen Inc. (Chatsworth, CA), and Q-Sepharose and benzamidine-Sepharose 4 Fast Flow was from GE Healthcare.

**Construction, Expression, and Purification of Recombinant Proteins**—A soluble form of hepsin comprising the entire extracellular domain was produced by use of a baculovirus expression system. A secreted His-tagged hepsin cDNA was constructed by fusion of the cDNA coding for the signal sequence of honeybee melittin (Met1-Tyr20) with the cDNA coding for the extracellular domain of human hepsin (Arg45-Leu417). The final cDNA construct was inserted in a baculovirus expression vector under the control of a polyhedrin promoter and expressed in T.in.Pro cells. Hepsin was purified by nickel-nitrilotriacetic acid affinity chromatography essentially as described (20). Hepsin-containing medium was conditioned with 1 mm sodium azide, 0.3 m NaCl, and 15 mm imidazole and was adjusted to pH 6.5 using NaOH. Precharged nickel-nitrilotriacetic acid resin was added to media (4 ml resin/liter medium). Batch absorption was performed by gently stirring at 4 °C for 2 h. After allowing the resin to settle for 1 h, the supernatant was decanted, and the resin was packed into a column. The column was washed with a minimum of 10 column volumes of PBS, 0.3 m NaCl, pH 7.4, then followed by 10 column volumes of 25 mm imidazole, 0.3 m NaCl, 1 mm sodium azide, pH 8.0. Proteins were eluted with 250 mm imidazole, 0.3 m NaCl, 1 mm sodium azide, pH 8.0. Pooled fractions were purified further using either ion-exchange chromatography on a Q-Sepharose FF ion or affinity chromatography on a benzamidine-Sepharose 4 Fast Flow column.

The matriptase protease domain was expressed in *Escherichia coli* and purified as described (20). HGFA and soluble HAI-2 were recombinantly expressed and purified as described (18, 20). The active site concentration of hepsin, matriptase, and HGFA was determined by using the potenti Kunitz domain inhibitor KD1 derived from HAI-1B, which was produced in *E. coli* as described (21). The active site concentrations were used for all enzymatic assays. For plasmin, the concentration provided by the supplier (Hematologic Laboratories, Inc.) was used.

**Monoclonal Anti-hepsin Antibody**—Five Balb/c mice (Charles River Laboratories) were hyperimmunized with recombinant soluble hepsin in Ribi adjuvant (Ribi Immunochechex Research Inc.). B cells from lymph nodes from five mice were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection, Manassas, VA) as previously described (22). After 10–14 days the supernatants were harvested and screened for antibody production with a hepsin binding enzyme-linked immunosorbent assay. The clone 3H10 showed high immunobinding and specificity after the second round of single cell per well cloning (Elite 1 Sorter, Beckman Coulter) and was scaled up for purification in INTEGRA CELLine 1000 (Integra Biosciences). The supernatant was purified by protein A affinity chromatography, sterile-filtered, and stored at 4 °C in PBS. Isotyping with the mono-AB-ID SP Kit (Zymed Laboratories Inc.) showed that 3H10 is an IgG1k.

**Production of Hepsin Overexpressing LnCaP Cells**—The human prostate carcinoma cell line, LnCaP-FGC (LnCaP), was obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 medium (American Type Culture Collection) plus 10% fetal bovine serum (Sigma-Aldrich). A LnCaP clone that stably expressed the firefly luciferase gene (LnCaP-luc) was used for hepsin transfection experiments. To establish the LnCaP-luc cell line, the luciferase gene was subcloned as an EcoRI/Xhol cDNA fragment inserted into the pMSCVneo expression vector (BD Biosciences Clontech, Mountain View, CA). LnCaP cells were transfected with the luciferase construct using Lipofectamine 2000 (Invitrogen). The cells were selected with 500 μg/ml Geneticin (Invitrogen), and clones were screened for bioluminescence activity by using the Luclite kit (PerkinElmer Life Sciences). The clone LnCaP-luc, which produced the strongest luminescence signal, was chosen for hepsin transfection experiments.

The cDNA of full-length hepsin was inserted into a mammalian expression vector containing the puromycin resistance gene for antibiotic selection (Genentech, South San Francisco, CA). The LnCaP-luc clone was transfected with the construct encoding full-length hepsin with a C-terminal FLAG tag, and the cells were selected with 0.5 μg/ml puromycin (Sigma-Aldrich). The clones were analyzed by fluorescence-activated cell sorter for hepsin surface expression using an anti-FLAG monoclonal antibody (Sigma-Aldrich). Two clones, the high hepsin expressor LnCaP-34 and the low hepsin expressor LnCaP-17, were selected for further experiments.

To measure total hepsin expression (endogenous and transfected) on the cell surface, LnCaP-34 and LnCaP-17 cell suspensions in PBS/1% (v/v) fetal bovine serum were incubated with 10 μg/ml of 3H10 antibody or without antibody (control) for 40 min on ice. The cells were washed twice with PBS before incubation with R-phycocerythrin-conjugated F(ab’2) goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.) diluted 1:1000 in PBS, 1% fetal bovine serum (v/v). After 30 min on ice the cells were washed with PBS, and cell pellets were resuspended in 1% formalin (Richard Allen Scientific). Antibody binding was measured on a FACScan (BD Biosciences).
Real-time Reverse Transcription-PCR—Total RNA was isolated from LnCaP-17 and LnCaP-34 cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Gene expression analysis was performed by real-time reverse transcription PCR (TaqMan) on a model 7500 sequence detector (ABI-PerkinElmer, Foster City, CA). To specifically measure endogenous hepsin we used primers recognizing sequences in the 3′-untranslated region of the hepsin gene. To measure total hepsin (endogenous plus transfected hepsin) we used primers recognizing sequences in the open reading frame that is common to both hepsins. The sequences of primers and probes were as follows: endogenous hepsin, forward (5′-CCCTCCAGGTCTCCTCCT-3′), reverse (5′-AGTCCCGAAGACAGGAACATAA-3′), probe (5′-(FAM)-CAGCCCCGAGACCCACCAAC-(TAMRA)-3′); total hepsin, forward (5′-GCTGTGGCATTGTGAGT-3′), reverse (5′-TGAGTCTTTATGGGCTGGGA-3′), probe (5′-(FAM)-AAGGCAGCCGTCTACACCAAATCGAC-(TAMRA)-3′); matriptase, forward (5′-CTTCCGAGCTCCTCAGT-3′), reverse (5′-GTCAGACCCGGCTCTGT-3′), probe (5′-(FAM)-CCTCCAGCTCCTCTCCT-3′); glyceraldehyde-3-phosphate dehydrogenase, forward (5′-GAAAGTGAAGGCTGAGTG-3′), reverse (5′-GAAGATGGTGATGGGATTTC-3′), probe (5′-(FAM)-CAGGCCTACACCAAATCGAC-(TAMRA)-3′). The reverse transcription was carried out at 48 °C for 30 min followed by heat activation of AmpliTaq Gold at 95 °C for 10 min. The thermal cycling proceeded with 40 cycles of 95 °C for 0.5 min and 60 °C for 1 min. All samples were run in duplicate. The results were quantified using the standard curve method according to the manufacturer’s instruction (ABI-PerkinElmer). All gene expression data was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Enzymatic Assays with HAI-2 and the Small Molecule Inhibitor HI-10331—The reversible active site inhibitor HI-10331 (a gift from Alan Olivero, Genentech, Inc.) or HAI-2 was incubated with 0.5 nm hepsin, 0.5 nm matriptase, and 10 nm uPA for 30 min in Hepes buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.01% Triton X-100) at room temperature. The chromogenic substrates S2366 (for hepsin), S2765 (for matriptase), and S2444 (for uPA) were added at a concentration corresponding to their respective K_m values, which were determined in separate experiments. After substrate addition, the linear rates of the increase in absorbance at 405 nm were measured on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

For HI-10331, the inhibitor concentration that gave a 50% inhibition of the enzymatic activity (IC_{50}) was determined by fitting the data to a four-parameter equation (Kaleidagraph Version 3.6, Synergy Software, Reading, PA). The K_v values were calculated according to the relationship K_v = IC_{50}/(1 + [S]/K_m) (23) using the experimentally determined IC_{50} and K_m values for each enzyme-substrate pair. For HAI-2, the apparent K_v values (K_v^*) were determined by fitting the data to the equation for tight binding inhibition (24, 25),

\[
\frac{v}{v_o} = 1 - \frac{([E] + [I] + K_v^*) - \sqrt{([E] + [I] + K_v^*)^2 - (4[E][I])}}{2[E]}
\]

(Eq. 1)

where v/v_o is the fractional activity, [E] is the enzyme concentration, and [I] is the inhibitor concentration.

Pro-uPA Activation by LnCaP Cells—Confluent LnCaP-34 and LnCaP-17 cells were washed with HBSA-glucose buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.05 mg/ml BSA, 5 mM glucose), and 0.8 ml of 100 mM pro-uPA in prewarmed HBSA-glucose buffer was added to the cell layers. The inhibitors KD1 or HI-10331 were added to give final concentrations of 1 and 10 µM, respectively. The culture plates were kept at 37 °C, and 50-µl samples were withdrawn at different time points and supplemented with 0.2 ml of 0.625 mM S2444 in Hepes buffer, and the increase in absorbance at 405 nm was measured on a kinetic microplate reader. Cell numbers were determined at the end of the experiments. The concentration of formed uPA in each sample was calculated from a standard curve of enzymatically converted pro-uPA and normalized to 10^6 cells. After subtracting the background levels of uPA formed in the absence of cell layer, the linear rates of uPA formation/10^6 cells were determined. The pro-uPA activation was strictly dependent on the presence of the cells, since the samples taken at different time points did not convert any additional pro-uPA. Also, the enzymatic activity toward S2444 of the withdrawn samples was entirely due to uPA activity and not to hepsin or other proteases released from the cell surface, since the chromogenic activity of the samples was not inhibited by the addition of the hepsin inhibitor KD1 but was completely inhibited by the uPA inhibitor PAI-1 (data not shown).

For immunoblotting experiments, confluent LnCaP-34 cell layers were washed as above and incubated in HBSA-glucose buffer with 30 nM pro-uPA at 37 °C in the presence of 1 µM KD1 or without. After 1, 3, and 5 h, aliquots were taken and immediately added to SDS sample buffer. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters using the Bio-Rad Semi-Dry Transfer system. Pro-uPA and uPA were visualized by using a rabbit polyclonal anti-uPA antibody (Cell Sciences, Canton, MA) followed by horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) and ECL (GE Healthcare) enhancement.

Analysis of Pro-uPA and Pro-tPA Activation by SDS-PAGE—Pro-uPA at a concentration of 1.5 µM was incubated with 15 nM hepsin or 15 nM plasmin in Hepes buffer at room temperature. After 4 and 60 min, aliquots of the reaction mixture were taken and added to 6× SDS sample buffer. The samples were analyzed by SDS-PAGE using a 4%–20% gradient gel (Invitrogen). Protein was visualized after staining with SimplyBlue Safe Stain (Invitrogen). Experiments with pro-tPA (1.5 µM) were carried out identically, except that the buffer used was 50 mM sodium phosphate, pH 7.5, 200 mM arginine, 0.01% Tween 20.

Determination of First-order Rate Constants—The K_m value for pro-uPA activation by plasmin is in the micromolar range (26–28). This is consistent with our findings in an attempt to determine K_m values for plasmin, hepsin, and matriptase. However, because the pro-uPA stock solution from the supplier (0.8 mg/ml) was not sufficiently high for accurate K_m measurements and the calculation of the catalytic efficiencies, we chose to determine the first-order rate constant k as a measure of pro-uPA conversion efficiency. It was ensured that the pro-uPA conversion used (30 nm) was in the range of first-order...
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kinetics for the enzymes tested, i.e. hepsin, plasmin, and matriptase. Pro-uPA (30 nM) was added to the enzymes (3 nM) in Hepes buffer to start the reaction at room temperature. At various time points, 50-μl aliquots were removed and added to 150 μl of 667 nM HAI-2 (final concentration in 250 μl was 400 nM) in Hepes buffer to stop further pro-uPA cleavage. At the concentration used, HAI-2 specifically inhibits plasmin (29), HGFA (30), hepsin, and matriptase but not the newly generated uPA (Table 1). Our results on HAI-2 inhibition of plasmin and HGFA (data not shown) were consistent with the published data (29, 30). After the addition of 50 μl of 2.5 mM S2444, the increase in absorbance at 405 nm was measured on a kinetic microplate reader. The concentration of uPA in each aliquot was determined, and the initial rates of plasmin formation were calculated.

Sample aliquots of uPA generated by hepsin (uPAHepsin), plasmin (uPAPlasmin), and respective controls. Aliquots of the reaction mixture were 3 nM hepsin or plasmin in Hepes buffer without pro-uPA. The controls were 3 nM hepsin or plasmin in Hepes buffer without pro-uPA. Sample aliquots of uPA generated by hepsin (uPAHepsin), plasmin (uPAPlasmin), and their respective controls were stored at −20 °C until further analysis.

For chromogenic assays thawed samples were 5-fold-diluted in Hepes buffer containing uPA chromogenic substrate S2444 (1–1000 nM) and 400 nM HAI-2 to inhibit hepsin and plasmin activities. The initial rates of S2444 cleavage were measured as change of absorbance at 405 nm on a kinetic microplate reader and expressed as μM concentrations of para-nitroanilide (pNA)/min using a pNA standard curve. The rates of pNA formation were presented as a function of S2444 concentration, and the Kₙ and Vₘₐₓ were determined after fitting the data to a 4-parameter equation (Kaleidagraph Version 3.6, Synergy software).

For plasminogen activation assays, a dilution-jump method was used. The reaction was started by adding 4 μM plasminogen to an equal volume of 25-fold diluted samples (uPAHepsin, uPAPlasmin, and respective controls). Aliquots of the reaction mixtures were taken at different time points and diluted 51-fold into 0.5 mm concentrations of the plasmin substrate S2366. Then the increase in absorbance at 405 nm was measured on a kinetic microplate reader. By use of a plasmin standard curve the concentration of plasmin in each aliquot was determined, and the initial rates of plasmin formation were calculated.

RESULTS

Cleavage of Pro-uPA by Hepsin Produces Enzymatically Active uPA—Incubation of pro-uPA with hepsin resulted in a time-dependent cleavage of the single-chain form into the two-chain form consisting of the 20-kDa A-chain and the 30-kDa B-chain (protease domain) (Fig. 1). Both chains remained disulfide-linked as shown by the single protein band of 55 kDa under non-reducing conditions (Fig. 1). The N-terminal sequence of the 20-kDa band was KSNELHQVPS (A-chain), and that of the 30-kDa band was 158VIGEFTTIEQ (B-chain), indicating that hepsin generated the high molecular weight form of uPA by processing pro-uPA at the consensus cleavage site Lys¹⁵⁸-Ile¹⁵⁹. In agreement, cleavage of pro-uPA by plasmin generated identical fragments and with a time course similar to hepsin. (Fig. 1). Extended incubation of pro-uPA with hepsin did not produce the 32-kDa low molecular weight form of uPA (under non-reducing conditions) (31) nor any visible degradation products. Pro-tPA, which is structurally related to pro-uPA, was only cleaved by plasmin but not by hepsin (data not shown).

To assay the enzymatic function of uPA generated by hepsin, pro-uPA was completely converted by hepsin (to give uPAHepsin) or by plasmin (to give uPAPlasmin) and then analyzed in two assay systems, i.e. cleavage of the small synthetic pNA substrate S2444 and of the macromolecular substrate plasminogen. First, the reaction velocity as a function of S2444 concentration was identical for uPAHepsin and uPAPlasmin (Fig. 2). The determined Kₙ values for uPAHepsin and uPAPlasmin were 34.1 ± 2.2 and 34.6 ± 1.5 μM (n = 3; ±S.D.), respectively, and the Vₘₐₓ values were 1.74 ± 0.51 and 1.70 ± 0.51 μM pNA min⁻¹ (n = 3; ±S.D.), respectively. Secondly, in plasminogen activation assays (Fig. 2B) the initial rates of plasmin generation by uPAHepsin and uPAPlasmin were 29.3 ± 5.0 and 26.4 ± 4.5 nM plasmin min⁻¹ (n = 3; ±S.D.), respectively. In both assay systems neither of the two controls (appropriately diluted hepsin and plasmin) had any activity (Fig. 2, A and B).

Enzyme Kinetics of Pro-uPA Activation by Hepsin, Plasmin, and Matriptase—To compare the efficiency of hepsin to convert pro-uPA with those of other known pro-uPA activators, such as plasmin and another member of the transmembrane serine protease family, matriptase (32, 33), we determined the first-order rate constants of pro-uPA activation. The use of the bi-Kunitz inhibitor HAI-2 allowed us to stop the reaction at different time points and to measure the time-dependent uPA generation, because HAI-2 is a potent inhibitor of plasmin (29), hepsin, and matriptase but not of uPA (Table 1) (29). HGFA (34), which is known to only activate pro-HGF, was used as a negative control. The results in Fig. 2 show the first-order reaction kinetics, indicating that hepsin and plasmin were equally active in processing pro-uPA, whereas matriptase cleaved pro-uPA at a significantly lower rate. During the exper-
TABLE 1
Equilibrium dissociation constants for the interaction of HAI-2 and the small molecule hepsin inhibitor HI-10331 with serine proteases

| Enzyme   | K_i (nM) ± S.D. | K_i (nM) ± S.D. |
|----------|----------------|----------------|
| Hepsin   | 0.26 ± 0.04    | 41.6 ± 1.1     |
| Matriptase| 0.85 ± 0.21   | >10,000        |
| uPA      | >1,000         | >10,000        |

* K_i, apparent equilibrium dissociation constant.

TABLE 2
Kinetic constants of pro-uPA activation by hepsin, plasmin, and matriptase

| Enzyme   | k (10^{-3} min^{-1} ± S.D.) | k_{cat}/K_m (10^3 M^{-1} s^{-1} ± S.D.) |
|----------|-----------------------------|----------------------------------------|
| Hepsin   | 87.1 ± 11.3                 | 4.84 ± 0.63                            |
| Plasmin  | 81.5 ± 14.2                 | 4.53 ± 0.79                            |
| Matriptase| 14.2 ± 4.9                  | 0.79 ± 0.27                            |

Pro-uPA Activation by Cell Surface-expressed Hepsin—To study pro-uPA processing on the cell surface, we established the LnCaP cell line LnCaP-34, which stably overexpressed full-length hepsin. Total hepsin RNA levels were about 9-fold higher than that of LnCaP-17, which only expressed endogenous hepsin as determined by RT-PCR TaqMan using specific primer/probe sets (Fig. 3A). Neither the endogenous hepsin expression nor matriptase expression was changed in the LnCaP-34 cells (Fig. 3A). Moreover, the monoclonal anti-hepsin antibody 3H10 allowed us to detect hepsin protein on the LnCaP cell surface. This antibody was raised against soluble hepsin extracellular domain and specifically recognized hepsin protein on LnCaP cells, but not on HPAC cells, which do not express any detectable hepsin mRNA (data not shown). The LnCaP-34 cells exhibited higher surface expression levels of hepsin compared with the LnCaP-17 cells, consistent with the different mRNA levels (Fig. 3B). The mean fluorescence intensity of the LnCaP-34 cells was 5-fold higher than the LnCaP-17 cells, indicating that LnCaP-34 expressed about 5-fold more hepsin on the cell surface.

The cell surface activity of hepsin was measured by incubating LnCaP-34 cells with 30 nM pro-uPA for 1, 3, and 5 h at 37 °C. Immunoblotting showed a time-dependent conversion of pro-uPA into the high molecular weight form as indicated by the appearance of a 30 kDa (B-chain) and 20 kDa (A-chain) band (Fig. 4A). The two chains were disulfide-linked as shown by the presence of a single 55-kDa band under non-reducing conditions, identical to the experiments with soluble hepsin (Fig. 1). The addition of the HAI-1B-derived Kunitz domain inhibitor KD1 (21) strongly inhibited pro-uPA cleavage (Fig. 4A).

The rates of uPA formation by cell surface hepsin were quantified with an enzymatic assay. To measure cell surface hepsin activity, pro-uPA was added to the cell layers, and the time-dependent formation of uPA was determined. Consistent with the different levels of hepsin expression, the rates of uPA formation were higher for LnCaP-34 compared with the LnCaP-17 (Fig. 4B, Table 3). The KD1 inhibitor almost completely inhibited pro-uPA cleavage by both cell lines (Fig. 4B, Table 3). Because KD1 does not inhibit uPA (21), it did not interfere with the determination of uPA concentrations in the second stage of the assay.

In addition to hepsin, both LnCaP-34 and LnCaP-17 express the pro-uPA activator matriptase (Fig. 3A), which can also be inhibited by KD1 (21). To find out whether matriptase activity contributed to uPA formation in this system, we used the small
molecule inhibitor HI-10331, which inhibited hepsin more than 200-fold more potently than matriptase but did not interfere with uPA activity (Table 1). We found that HI-10331 reduced the pro-uPA activation rates by the hepsin-overexpressing LnCaP-34 almost to the same level as KD1 (Table 3), indicating that, compared with hepsin, there was little pro-uPA-converting activity by matriptase (<10%). On the other hand, in LnCaP-17 cells, which had an about 3-fold lower pro-uPA converting activity, the potential contribution by matriptase was more substantial, since the rate of pro-uPA cleavage remained at about 30% of the control rate in the presence of HI-10331 (Table 3).

**DISCUSSION**

The herein reported pro-uPA converting activity of hepsin provides a molecular basis to link hepsin overexpression with prostate cancer progression. Hepsin itself does not directly convert plasminogen to plasmin (17, 18) but instead amplifies plasmin generation by activating pro-uPA. uPA proteolytically cleaves plasminogen to plasmin, which in turn degrades components of basement membranes and the extracellular matrix either directly or indirectly by activating latent matrix metalloproteases (43, 44). The association of the plasminogen activation system with tumor invasion and dissemination is well documented (43, 44). Hepsin also activates the latent form of the invasive growth factor HGF (17, 18), and both HGF and uPA have been implicated in prostate cancer growth and metastasis (45–48). Moreover, by activating pro-HGF, hepsin may directly regulate the local levels of its substrate pro-uPA since HGF was shown to induce pro-uPA transcription and protein synthesis (49, 50). Therefore, tumor cell surface-expressed hepsin could be central in orchestrating invasive pathways at the tumor/stroma interface. Such a concept is consistent with the hepsin-mediated tumor progression and the associated basement membrane disruption in a mouse model of prostate cancer (16).

The ability of hepsin to activate pro-uPA as well as pro-HGF (17, 18) is akin to the transmembrane serine protease matriptase (20, 32, 33), which like hepsin is overexpressed in
prostate and ovarian cancer (51–55). Therefore, hepsin and matriptase may engage in overlapping functions that contribute to tumor progression. Their increased expression in prostate and ovarian tumors is not paralleled by HAI-1 (53–55), a bi-Kunitz inhibitor that is also expressed on tumor epithelium and potently inhibits both enzymes in vitro (17, 18, 20, 56). In fact, the HAI-1 expression levels during prostate cancer progression were reported to remain unchanged (54) or to be reduced (55). In either case this would result in an enzyme: inhibitor imbalance that might favor tumor progression. An intriguing example that illustrates the consequences of an unbalanced enzyme:inhibitor system is matriptase overexpression in the mouse skin epidermis, where normally matriptase and its physiologic inhibitor HAI-1 are co-expressed in a regulated manner. Matriptase overexpression resulted in the spontaneous formation of neoplastic lesions, which could be completely prevented by the simultaneous overexpression of the inhibitor HAI-1 (57). Clearly, there are important functional differences between matriptase and hepsin. First, unlike matriptase, the in vivo overexpression of hepsin in mouse prostate did not lead to spontaneous neoplasia (16). Secondly, matriptase is more widely expressed in normal and tumor tissues and engages in physiologic functions such as skin epidermal differentiation (58), in which hepsin is not involved. Nevertheless, the concerted up-regulation of both proteases in prostate and ovarian cancer combined with their similar substrate specificities suggests a functional cooperation in cancer progression.

Hepsin specifically cleaved pro-uPA at the consensus Lys\(^{158-159}\) Ile\(^{159}\) peptide bond to produce high molecular weight uPA, which was identical to the reference uPA material in respect to small synthetic and macromolecular substrate activation. Thus, hepsin converted pro-uPA into fully functional uPA with respect to both catalysis and uPA receptor binding, since high molecular weight uPA contains the receptor binding site located in the N-terminal epidermal growth factor and Kringle domains (59). Because the enzymatic assays were carried out with a soluble form of hepsin, it was important to assess whether pro-uPA conversion was recapitulated by full-length hepsin on the cell surface. Using the hepsin overexpressing LnCaP-34 cell line, we were able to demonstrate that like soluble hepsin, the cell surface-expressed hepsin converted pro-uPA into high molecular weight uPA. In agreement with the higher hepsin protein expression, the rate of uPA formation by LnCaP-34 cells (6.6 nm uPA h\(^{-1}\) ± S.D.) was about 3-fold higher than the LnCaP-17 cells, which expresses about 5-fold less hepsin on the surface. Matriptase, which is also expressed by LnCaP-17 cells, did not significantly contribute to uPA generation. The lower matriptase activity toward pro-uPA in the cell-based system agrees well with the about 6-fold lower catalytic efficiency determined in purified enzyme assays.

The catalytic efficiency of hepsin for pro-uPA conversion was remarkably high (\(k_{cat}/K_m = 4.8 \times 10^8\) m\(^{-1}\) s\(^{-1}\)) and is similar to plasmin (Table 2 (26–28)), which is considered the most potent pro-uPA activator. This could imply that hepsin is an alternative pro-uPA activator equivalent to plasmin in respect to efficiency. Hepsin, thus, differs from less potent pro-uPA convertases, which may only trigger the plasminogen/plasmin system by generating trace amounts of plasmin to allow for the efficient feedback activation of pro-uPA.

Except for pro-uPA, all known macromolecular substrates of hepsin have an Arg at the P\(_1\) position (17–19), which is consistent with the results of a peptide library screening study that showed a strong preference of Arg over Lys as the P\(_1\) residue (17). Yet the dual recognition of macromolecular substrates with Arg or with Lys in P\(_1\) is not without precedent for trypsinlike serine proteases, as exemplified by plasmin and plasma kallikrein. A molecular model of the P\(_4\)-P\(_1\) sequence of pro-uPA (Pro-Arg-Phe-Lys) could be built without major difficulties using the published hepsin structure with the tetrapeptide Lys-Gln-Leu-Arg-chloromethylketone (17), derived from pro-HGF, in the active site (data not shown). The simple fit of Pro-Arg-Phe-Lys into this hepsin structure is imperfect and would be consistent with a reduced affinity compared with the pro-HGF sequence. However, the substrate binding clefts of trypsin-family enzymes are not rigid, and a low energy conformational adjustment that would better match the pro-uPA sequence is very probably available. Indeed, given the rapid turnover of pro-uPA by hepsin, a suitable accommodation must be made.

In conclusion, the activation of pro-uPA by hepsin provides a mechanistic basis to explain the basement membrane disorganization observed in the hepsin-overexpressing mouse prostate (16). The recent finding, although based on a small number of samples, that hepsin protein is strongly expressed in bone metastatic lesions and advanced primary prostate cancer (12), may indicate a role of hepsin during the later stages of disease. This would agree with the requirement of motility factors (e.g. HGF) and matrix degradation (uPA/plasmin), which could be influenced by hepsin enzymatic activity. Whether hepsin plays a role in the early stages of prostate cancer requires more experimental evidence. The recognition that hepsin is a potent activator of pro-uPA and pro-HGF in vitro provides a conceptual framework to specifically investigate these questions in vivo. The recent development of potent protein-based inhibitors of hepsin, such as monoclonal antibodies (12) and HAI-1-derived Kunitz-domain inhibitor (21), may help unraveling hepsin’s role in cancer.

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