Identification of Hepatocyte Growth Factor Activator Inhibitor-1B as a Potential Physiological Inhibitor of Prostasin*

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Prostasin is a trypsin-like serine protease that is glycosylphosphatidylinositol-anchored to the epithelial cell surface, from where it can be released in a soluble form. We undertook a co-expression search using the Genesis Enterprise System Database from Gene Logic to identify prostasin inhibitors, on the assumption that prostasin and its natural inhibitors may have a similar gene expression pattern. We found the expression profile of prostasin in normal human tissues to correlate highly with hepatocyte growth factor activator inhibitor-1B (HAI-1B) and its splice variant HAI-1. Soluble HAI-1B (sHAI-1B), comprising the entire extracellular domain, formed a 1:1 complex with purified prostasin in protein binding assays and inhibited prostasin enzymatic activity with an IC50 of 66 ± 15 nM. Two sHAI-1B mutants with inactivated N- and C-terminal Kunitz domains (KD1 and KD2) were used to show that the interaction of sHAI-1B with prostasin is mediated by KD1. In agreement, KD1 (Thr246-Val303) alone potently inhibited prostasin activity (IC50 = 4.7 ± 0.5 nm). Furthermore, prostasin was isolated with two major HAI-1/1B fragments (40 and 58 kDa) from OVCAR3 cell medium, demonstrating that prostasin-HAI-1/1B complexes are formed naturally. Moreover, when prostasin and HAI-1B were co-expressed in Chinese hamster ovary cells, complexes of prostasin with HAI-1B were detected on the cell membrane as well as in the culture medium, suggesting that preformed complexes were shed from the cell surface. The identification of HAI-1B as a potential physiological regulator of prostasin function, as described herein, may further the investigation of the role of prostasin in normal physiology and cancer.

Prostasin is a glycosylphosphatidylinositol-anchored, trypsin-like serine protease expressed on the surface of epithelial cells (1, 2). Mature prostasin is generated by a specific cleavage between Arg12 and Ile13, and consists of a 12-amino acid light chain disulfide linked to a 299-amino acid protease domain (3). Prostasin was first purified as a soluble enzyme from human seminal fluid, suggesting that the membrane-anchored form undergoes shedding (1). The physiological functions and substrates of prostasin remain unclear. Recent studies suggest that it may play a role in regulating the epithelial sodium channel in airway epithelia (4, 5). Using microarray technology, Mok et al. (6) found increased prostasin expression in cancerous ovarian epithelial cells and suggested that prostasin, in combination with cancer antigen-125, could be a potential serum marker for ovarian cancer. In contrast, prostasin expression is significantly down-regulated in high grade or hormone-refractory prostate tumors and is lost in highly invasive human and mouse prostate cancer cell lines (7, 8). It was proposed to function as an invasion suppressor, because the transfection with prostasin cDNA reduced in vitro invasiveness of the prostate carcinoma cell lines DU-145 and PC-3 (7) as well as the breast cancer cell lines MDA-MB-231 and MDA-MB-435s (9). A prostasin-binding protein was found in human and mouse seminal vesicle fluid and identified as protease nexin-1 (PN-1)†‡ (2, 10), a secreted serine protease inhibitor expressed in a variety of cell types (11). PN-1 forms an SDS- and heat-stable complex with prostasin and exhibits inhibitory activity toward prostasin with unclear potency (10). However, PN-1 does not form a stable complex with prostasin in the extracts of some prostasin-abundant tissues such as prostate, lung, and salivary glands (2), suggesting that additional prostasin inhibitors may exist.

Hepatocyte growth factor activator inhibitor-1B (HAI-1B) is a recently identified splice variant of HAI-1, a b1-Kunitz-type serine protease inhibitor found mainly in epithelium (12–14). HAI-1 and HAI-1B (referred to as HAI-1/1B) are thought to be involved in tissue regeneration and tumorigenesis by inhibiting the activation of pro-HGF. Deletion of the HAI-1 gene resulted in embryonic lethality due to severely impaired formation of the placental labyrinth layer (15). Enhanced expression of HAI-1 was noted in regenerating epithelial cells such as the regenerative colon epithelium of acetic acid-induced mouse colitis models (16), indicating a possible role in regulating the level of HGF activation. In agreement with this hypothesis, HAI-1 expression in colorectal mucosa was down-regulated in adenocarcinoma, where pro-HGF processing is enhanced (17, 18). HAI-1/1B consists of an N-terminal Kunitz domain (KD1), a low density lipoprotein receptor-like domain, a C-terminal Kunitz domain (KD2), a transmembrane domain, and a cytoplasmic domain (14). KD1 is mainly responsible for the inhibitory activity according to mutagenesis studies (13, 19). HAI-1/1B is synthesized as a transmembrane protein on the cell surface and appears to be subsequently shed (14). The membrane-bound HAI-1 is able to form a complex with active HGF activator (HGFA) and may function to temporarily sequester HGFA to the cell surface and serve as a reservoir for HGFA (20). The shed form of HAI-1/1B is active in inhibiting HGFA (14), matriptase (21), and hepasin (22, 23), all of which are pro-HGF activators. In addition, matriptase was found to activate single chain urokinase-type plasminogen activator (24, 25), which degrades the extracellular matrix and plays a critical role in tumor invasion. HAI-1B differs from HAI-1 by a 16-amino acid insertion after the first Kunitz domain (13). No significant differences between the two splice variants

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HAI-1B Is an Inhibitor of Prostasin

have been found in respect to tissue distribution, enzymatic activity, or specificity.

In this study, we attempted to identify physiological prostasin inhibitors by analyzing gene expression data with the assumption that prostasin and its cognate inhibitor(s) are co-expressed. This assumption is supported by previous experimental work showing co-expression of other pairs of effector molecules and their endogenous inhibitors. For example, granzyme B has been found to be expressed in the same cells as its inhibitor PI-9 (SERPINB9) in humans (26) and Spi6 in mice (27), which may play a protective role in host cells against the proteolytic effects of the serine protease. Similarly, quantitative correlation of expression has been found between matrix metalloproteinase 1 (MMP-1) and its inhibitor TIMP-1 in myocardium (28), which is thought to serve as a mechanism to regulate the activity of MMP-1.

Using the co-expression analysis, we identified HAI-1/1B as the highest-ranking prostasin inhibitor and verified its interaction with prostasin experimentally. We show that prostasin enzymatic activity is inhibited by a soluble form of HAI-1B (sHAI-1B). The interaction of prostasin and HAI-1B in solution and in cell culture was also studied. Our results suggest that HAI-1B is a potential physiological inhibitor of prostasin.

MATERIALS AND METHODS

Reagents—sHAI-1B and its mutants, sHAI-1B(R260A) and sHAI-1B(K401A), were expressed and purified as described previously (13). Soluble HAI-1 protein (sHAI-1) comprising the entire extracellular domain was obtained from R&D Systems (Minneapolis, MN). KDI (Thr246-Val303 of HAI-1B precursor) was expressed in Escherichia coli and purified as described (29). Aprotinin-agarose was purchased from Sigma, aprotinin from Roche Applied Science, chromogenic substrate and purified as described (29). Aprotinin-agarose was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alzheimer amyloid-β protein precursor inhibitor and KALI-DY (30) were generously provided by Mark Dennis (Genentech, Inc.).

Screening for Protease Inhibitors Co-expressed with Prostasin—For our source of gene expression data, we used the Genesis Enterprise SystemTM data base (Gene Logic Inc., Gaithersburg, MD) containing gene expression signatures from over 8000 clinical samples from various normal and disease conditions on the Affymetrix HG-U133A and B GeneChip® microarrays. From this data base, each probe set on the microarray has a gene expression profile consisting of the expression results of each of the samples. The gene expression profile can be considered as a vector of expression values, one value for each sample. We limited our analysis to samples from epithelium-containing tissues labeled in the data base as normal and to those tissues that contained more than 20 normal samples. For each of these tissues, we performed a search starting with the gene expression profile for prostasin (Affymetrix probe set 202525_at), comparing it with the profiles for each of the other probe sets. The comparison of a given probe set with that of prostasin can be visualized as a scatter plot, with the expression value of prostasin on the x axis and the expression value of the given probe set on the y axis, and each sample from the given tissue represented by a point in the scatter plot. From this scatter plot, the co-expression of the given probe set with prostasin can be measured by the Pearson correlation coefficient r. High values of r indicate probe sets with a gene expression profile similar to the starting probe set (31). We sorted all probe sets by their value of r and then filtered these lists to identify protease inhibitors that had high correlation coefficients. Probe sets corresponding to putative protease inhibitors were obtained by searching the NetAffx data base (32) for those annotated as belonging to a gene ontology molecular function of protease inhibitor activity (GO identification 0030414) or one of its subtypes (0042031, 0030415, 004866, 0019828, 0004869, 0030161, 0008191, 0004867, 0053659, 0030568, 0030304) (www.geneontology.org). This search yielded 177 Affymetrix probe sets on the HG-U133A and HG-U133B microarrays.

Reverse Transcription-PCR—Total RNAs from various human tissues were purchased from BD Biosciences Clontech (Palo Alto, CA). RNA from OVCAR3 cells was extracted using the RNeasy Kit (QIagen, Valencia, CA). These total RNAs were processed by using oligo(dT)24 and SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNAs and template controls were subjected to PCR using the primer set of prostasin, HAI-1, HAI-1B, or β-actin (as a control). The sequences of these primers were as follows: 5'-CCTGGGGCCCTGGG-CAGC-3' and 5'-TGGCGGCTCTCAGGTTGG-3' for prostasin; 5'-ATGGAGGCTGTCTGGGCAACA-3' and 5'-ACAGGGACGCTCTG-TCGGAGG-3' for HAI-1 and HAI-1B; 5'-TCACCCACACTGTGGCC-CATCTCGA-3' and 5'-CAGCGAACTGCTATTGGAATG-G-3' for β-actin. The PCR amplifications were carried out for 25 cycles of 45 s at 95 °C, 45 s at 55 °C (for HAI-1 and HAI-1B), or 65 °C (for prostasin) and 1 min at 72 °C using Advantage-GC cDNA polymerase mix (BD Biosciences-Clontech). The reverse transcription-PCR products were separated on a 2.5% agarose gel and then visualized by ethidium bromide staining.

Cloning, Expression, and Purification of Prostasin—Full-length human prostasin cloned from a human cDNA library was inserted into the eukaryotic expression vector pRK5E. Recombinant protein was produced using a transient transfection process in CHO cells. Briefly, the cells were transfected with DNA-cationic lipid complex preformed for 15 min in basal medium and were then grown in 3-litter spinner flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mg/liter recombinant human insulin and trace elements. The culture was maintained at 33 °C for 5 days. The conditioned medium was harvested and loaded onto an aprotinin-agarose column equilibrated with 20 mM Tris-HCl, pH 7.5. After washing with the same buffer, prostasin was eluted with 100 mM glycine, pH 3.0, containing 150 mM NaCl. The eluates were neutralized by 0.5 M Tris immediately. Pooled prostasin fractions were concentrated and dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl. N-terminal amino acid sequence analysis gave a sequence of H11TGSSAVAGQW24, indicating that purified prostasin was in the active two-chain form. Protein concentration was determined by use of the bicinchoninic acid assay (Pierce).

Generation of Antibodies—To obtain polyclonal antibodies against sHAI-1B or prostasin, 0.3 mg of recombinant protein was injected into each rabbit (Antibody Solutions, Palo Alto, CA). After 9–11 weeks, the rabbit sera with the highest titers were pooled and antibodies purified by protein A affinity chromatography. Mouse monoclonal antibody 6E9 against sHAI-1B was generated using standard hybridoma-producing techniques (33). Hybridoma cell lines secreting antibody specific for sHAI-1B, as determined by enzyme-linked immunosorbent assay, were cloned twice by limiting dilution and further characterized. The monoclonal antibody was then purified from ascites fluid by protein A affinity chromatography. The polyclonal and monoclonal anti-HAI-1B antibodies reacted with both HAI-1 and HAI-1B (data not shown).

Immunoblotting—Proteins were separated on a 4–20% SDS-PAGE and transferred to nitrocellulose membrane (Invitrogen). After blocking the nonspecific binding sites with 2% bovine serum albumin in PBS, the membrane was incubated with primary antibody followed by incubation with 1:40,000 dilution of a goat anti-mouse or a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), which was detected by use of the
ECL Plus system (Amersham Biosciences). The primary antibodies used were as follows: polyclonal anti-HAI-1B antibody (4 μg/ml), monoclonal anti-HAI-1B antibody (3 μg/ml), monoclonal anti-prostasin antibody (1:500, BD Biosciences).

Prostasin Binding Assay—Purified prostasin and sHAI-1B were diluted in 20 mM Tris-HCl, pH 9.0, containing 0.01% Triton X-100 (TT buffer) to the final concentration as indicated. The mixture was incubated at 37 °C for 0.5 or 2 h and SDS sample buffer with dithiothreitol was added. Some of the samples were boiled for 5 min as indicated in Fig. 2. 30 μl of each sample was analyzed by immunoblotting.

Enzyme Inhibition Assay—Inhibitors were incubated with prostasin (final concentration, 3 mM) in 225 μl of TT buffer for 20 min at room temperature before the addition of 25 μl of 0.5 mM S2765 (final concentration, 0.05 mM; Ki, 0.05 mM). The increase in absorbance at 405 nm was monitored on a kinetic microplate reader ( Molecular Devices, Sunnyvale, CA). The linear rates of the increase in absorbance were expressed as percentage activities (100% × v/v0). Inhibitor activity was calculated as the concentration of inhibitor giving 50% inhibition (IC50) of the uninhibited enzyme activity. At least three independent experiments were performed for each inhibitor.

Transfection of CHO Cell Lines—Full-length HAI-1B was cloned by PCR as described previously (13) and inserted into the expression vector pRK5E. HAI-1B, or vector alone was transferred into CHO cells using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. An HAI-1B-expressing clone was selected by immunoblotting the cell membrane. HAI-1B, and vector stably transfected CHO cell lines were designated CHO/HAI-1B and CHO/vector, respectively.

HAI-1B Ectodomain Shedding Assay—CHO/HAI-1B and CHO/vector cells were grown to 50% confluence in DMEM plus 10% serum in 6-well plates. Cells were washed with PBS and grown to 90% confluence in serum-free medium supplemented with 2 mg/liter recombinant human insulin and trace elements. Then 1 ml of medium containing 200 ng/ml prostasin or buffer (controls) was added. After continued incubation for 2 or 24 h, 300 μl of medium was collected and centrifuged at 5,000 rpm for 5 min on a desktop microtube centrifuge. The supernatant was mixed with sample buffer containing dithiothreitol and then concentrated by a Microcon® centrifugal filter device (YM-10) (Millipore, Billerica, MA) to 30 μl. Samples were boiled and analyzed by immunoblotting. To prepare cell lysates, cells were incubated for 24 h, washed with PBS, and incubated with 300 μl of M-PER™ lysis buffer (Pierce)/well for 5 min at room temperature. Then the cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C. 10 μl of supernatant was used for immunoblotting analysis.

Isolation and Detection of the prostasin/HAI-1/1B Complexes from Cell Culture—CHO/HAI-1B and CHO/vector cells were grown as described for the HAI-1B ectodomain shedding assay, except that they were grown in 10-cm dishes. When cells reached 90% confluence, 3 ml of fresh serum-free medium with or without 200 ng/ml prostasin was added. After 2 h, the medium was harvested, and the cells were washed and lysed with 1 ml of M-PER™ lysis buffer. The transient transfection of CHO/HAI-1B and CHO/vector cells by pRK5E vector bearing full-length prostasin was performed in 10-cm dishes using FuGENE 6 transfection reagent (Roche Applied Science). When cells reached 70% confluence, 3 ml of fresh serum-free medium was added. The medium was collected after 48 h. The cells were washed and removed by trypsin treatment (0.25% with 1 mM EDTA). Cell membranes were prepared by resuspending the cells in 1 ml of cold Tris-HCl buffer (10 mM Tris-HCl, pH 7.5). Cells were homogenized with a Duall homogenizer followed by centrifugation at 1,300 × g for 10 min at 4 °C.

The supernatant was centrifuged at 14,000 × g for 30 min at 4 °C. Then the pellet was resuspended with 300 μl of M-PER™ lysis buffer (Pierce) and incubated for 15 min at room temperature. Insoluble fractions were removed by centrifugation, and the soluble fractions contained membrane extracts.

OVCAR3 cells were grown to 90% confluence in 250 ml DMEM plus 10% serum in a 2-liter roller bottle. All of the 250-ml medium was used in the purification of the complexes.

Antibodies were immobilized to Aminolink® Plus Coupling Gel (Pierce) according to manufacturer’s protocol. The coupling efficiency was determined by measuring the protein concentration of the starting material and the non-bound fraction. The amount of coupled antibody ranged from 1.4 to 2.3 mg/ml resin. Prostasin/HAI-1B complexes were purified by incubation of medium or cell extract with antibody-coupled resin (containing 100 μg of antibody) at 4 °C with end-to-end rotation overnight. The resin was then washed with PBS and the bound proteins eluted with 60 μl ImmunoPure® IgG elution buffer (Pierce). For isolation of the complexes from OVCAR3 cell culture, the cell medium was loaded onto a Poly-Prep column (Bio-Rad Laboratories) pre-packed with the antibody-coupled resin (containing 350 μg of antibody 6E9) at room temperature. The resin was then washed with PBS and the bound proteins eluted with 500 μl of ImmunoPure® IgG elution buffer. SDS sample buffer with dithiothreitol was added to the eluates and boiled for 5 min before each sample was analyzed by immunoblotting.

RESULTS

Expression Profiles of Prostasin and HAI-1/1B—We undertook a co-expression search to find prostasin inhibitors on the assumption that natural inhibitors may have gene expression patterns similar to those of their target enzymes. The 177 probe sets used represented 111 different inhibitors of all major protease inhibitor families. Data analysis revealed HAI-1 (represented by Affymetrix probe set 202826_at) as a high ranking protease inhibitor in normal tissues (TABLE ONE). HAI-1 ranked highest in 7 and second highest in 6 of the 17 examined tissues. The p values for these tissues were all statistically significant (p < 0.005). In cases where HAI-1 was not the highest ranking protease inhibitor, the table shows the protease inhibitor that did rank highest. In three cases where HAI-1 ranked second (cervix, endometrium, and esophagus), the highest ranking protease inhibitor was HAI-2, a bi-Kunitz-type serine protease inhibitor structurally related to HAI-1.

The Affymetrix probe sets for HAI-1 were from the 3’-untranslated region; therefore, the expression data reflected the combined expression of both splice variants HAI-1 and HAI-1B. To determine whether prostasin expression correlates with both or either of the isoforms, the expression of prostasin, HAI-1, and HAI-1B was examined in 21 tissues by reverse transcription-PCR (Fig. 1). The results showed that in all tissues with high prostasin expression (lung, placenta, prostate, salivary gland, thyroid gland, trachea, colon, and small intestine) both HAI-1 isoforms were highly expressed as well. These findings are in full agreement with the Affymetrix microarray data showing that HAI-1/1B ranked as the highest or second highest protease inhibitor in these prostasin-abundant tissues.

1:1 Complexes of Prostasin and sHAI-1B—Soluble prostasin was purified from conditioned medium of CHO cells transiently transfected with full-length prostasin. N-terminal sequencing and SDS-PAGE analysis of purified prostasin indicated that activation cleavage at Arg12-Ile13 had occurred, yielding the enzymatically active two-chain form. sHAI-1B comprising the entire extracellular domain of HAI-1B (13) was incubated with prostasin at different molar ratios (Fig. 2A). Increasing concentrations of prostasin shifted the sHAI-1B band (55 kDa) to the
higher molecular mass species of 85 kDa, indicating the formation of prostasin/H1A1B complexes. At a 10-fold molar excess of prostasin, almost all sHAI-1B was bound to prostasin. Immunoblotting with anti-prostasin antibody confirmed that the 85-kDa band comprised complexes of sHAI-1B and prostasin (data not shown). Heat denaturation of the complex produced the expected sHAI-1B band (58 kDa) indicating that prostasin, even at molar excess, does not degrade sHAI-1B. The apparent molecular mass of 85 kDa indicated that prostasin and sHAI-1B formed a 1:1 complex. We did not detect any higher molecular mass complexes, suggesting that only one of the two sHAI-1B Kunitz domains engaged in prostasin binding. To identify the Kunitz domain interacting with prostasin, we utilized two sHAI-1B mutants in which the Kunitz domains were separately inactivated by changing the P1 residues Arg260 (in KD1) and Lys401 (in KD2) to Ala (13). As shown in Fig. 2B, the KD2 mutant sHAI-1B(K401A) still formed an 85-kDa complex with prostasin, whereas the KD1 mutant sHAI-1B(K260A) did not. These results suggested that sHAI-1B binds to prostasin through interaction with KD1.

Inhibition of Prostasin Activity by sHAI-1B—In screening a panel of synthetic para-nitroanilide substrates, we identified N'-benzoyl-L-Arg-Gly-Arg-pNA-2HCl (S2765) as the most suitable prostasin substrate, and it was used for subsequent inhibition assays. Wild-type sHAI-1B inhibited the amidolytic activity of prostasin toward S2765 with an IC₅₀ value of 66.0 ± 15.2 nM (TABLE TWO). In agreement with the binding data, sHAI-1B(R260A) was at least 18-fold less potent (IC₅₀ 1000 nM), whereas sHAI-1B(K401A) showed inhibitory potency comparable with the wild type. Furthermore, the 58-amino acid
KD1 (Thr^{246}-Val^{250}) potently inhibited prostasin enzymatic activity with an IC_{50} of 4.7 ± 0.5 nM. The other splice variant, shAI-1, inhibited prostasin with a potency comparable with shHAI-1B (TABLE TWO). Other examined Kunitz domains, such as Alzheimer amyloid-β protein precursor inhibitor and KALI-DY (30), did not inhibit prostasin (TABLE TWO). Aprotinin inhibited prostasin with an IC_{50} of 1.2 ± 0.1 nM, similar to the value obtained by Yu et al. (1) using prostasin purified from human seminal fluid.

**Interaction of Prostasin with HAI-1B in Cell Culture**—To determine whether HAI-1B binds to prostasin under more physiologic conditions, we examined whether endogenous prostasin/HAI-1B complexes are formed in cell culture. RNA expression experiments showed that the ovarian carcinoma cell line OVCAR3 expressed prostasin as well as both HAI-1 isoforms (Fig. 3A). Expression of the proteins was confirmed by immunoblotting experiments using OVCAR3 cell lysate (Fig. 3B). The 64-kDa band detected by the polyclonal anti-HAI-1B antibody probably represented both isoforms, because the calculated molecular masses of HAI-1 and HAI-1B only differed by 1.5 kDa. Immunoaffinity purification of conditioned medium with 6E9, a monoclonal antibody raised against HAI-1B, yielded two major HAI-1/1B fragments with molecular masses of 58 and 40 kDa (Fig. 3C). The antibody 6E9 was immunoreactive with both shHAI-1B and shHAI-1 (data not shown), and therefore the two fragments observed could be derived from either or both isoforms.

Probing the blot with an anti-prostasin antibody identified a 38-kDa prostasin band (Fig. 3C), indicating that HAI-1B and prostasin spontaneously formed endogenous complexes. The slightly lower mass of prostasin from OVCAR3 medium versus prostasin isolated from transiently transfected CHO cells (Fig. 3C, lane 2 versus lane 3) is possibly due to differences in glycosylation (1, 5) or cell type-specific proteolytic processing.

### TABLE TWO

| Inhibitors | IC_{50} [nM] |
|------------|-------------|
| shHAI-1    | 66.0 ± 15.2 |
| shHAI-1B(K401A) | 75.3 ± 12.4 |
| shHAI-1B(R260A) | >1000   |
| KD1        | 4.7 ± 0.5  |
| shHAI-1    | 109.0 ± 9.2 |
| APP\(^{\text{i}}\) | >1000   |
| KALI-DY    | >1000      |

\(^{\text{a}}\) The IC_{50} values are the averages ± S.D. of at least three independent experiments.

\(^{\text{b}}\) Alzheimer amyloid-β protein precursor inhibitor.

To further understand the interaction of prostasin with HAI-1B, a stable CHO cell line over-expressing full-length HAI-1B was established. We found that surface-expressed HAI-1B (64 kDa) was spontaneously cleaved, releasing identical amounts of the two major HAI-1B fragments into the medium (Fig. 4A). The molecular masses of these fragments, 58 and 40 kDa, were identical to those found in OVCAR3 cell medium (Fig. 3C). No HAI-1B was detected in the CHO/vector cells (Fig. 4A). Both shed HAI-1B fragments were detected as early as 2 h, and further incubation resulted in more HAI-1B shedding without changes in the ratio of the two species. To determine whether prostasin forms complexes with shed HAI-1B, the CHO/HAI-1B cells were incubated with prostasin for 2 h followed by immunoprecipitation from the conditioned medium using monoclonal antibody 6E9. The addition of prostasin slightly induced HAI-1B shedding (2–3-fold induction, data not shown) without changing the relative amounts of the 58- and 40-kDa HAI-1B fragments (Fig. 4B, upper panel). Prostasin was detected along with immunoprecipitated HAI-1B, indicating that it formed complexes with the shed HAI-1B (Fig. 4B). Because both the 58- and 40-kDa forms were released into the medium, the question arose as to which molecular species was engaged in complex formation. To answer this question, a similar experiment was performed using the anti-prostasin antibody to co-immunoprecipitate bound HAI-1B fragment. Both the 58- and 40-kDa forms were detected on immunoblots (Fig. 4C), with the 40-kDa form consistently giving the stronger signal compared with the 58-kDa form. This indicated that both HAI-1B fragments are capable of binding prostasin.

Moreover, to demonstrate that prostasin is able to interact directly with HAI-1B on the cell membrane, CHO/HAI-1B cells were incubated with soluble prostasin, and the formed complexes co-immunoprecipitated from cell lysate with an anti-HAIB antibody. The results showed that prostasin specifically bound to membrane HAI-1B (Fig. 4D). To further confirm their interaction on the cell surface, full-length prostasin and HAI-1B were co-expressed in CHO cells. First, as illustrated in Fig. 4E, transient transfection of CHO/vector and CHO/HAI-1B cells with full-length prostasin resulted in similar levels of membrane-expressed prostasin protein. Second, by using the anti-HAI-1B antibody 6E9, prostasin/HAI-1B complexes were immunoprecipitated from the cell membrane fraction and were detected as shed forms in the culture medium (Fig. 4F). The higher intensity of the prostasin band of the secreted prostasin/HAI-1B complex is simply a reflection of the large amounts of medium used for these experiments. On a cell number basis, the prostasin complexes secreted into the medium during a 48-h period represent only a small portion of the membrane-associated prostasin.

**FIGURE 3.** Prostasin forms complexes with HAI-1B in OVCAR3 cell medium. A, reverse transcription-PCR of total mRNA was carried out to examine the expression of prostasin, HAI-1, and HAI-1B in OVCAR3 cells. rt, no template control; tpl, template control using vectors bearing full-length prostasin, HAI-1B, or HAI-1; ovc, OVCAR3 cells. B, expression by OVCAR3 cells of HAI-1/1B and prostasin protein was detected by the indicated antibodies. ovc, 30 µg of OVCAR3 cell lysate; ctr, 10 ng of shHAI-1B (upper panel) or 25 ng of purified soluble prostasin (lower panel); cho, 30 µg of CHO cell lysate as negative control. Ib, immuno- blotting. C, OVCAR3 cells were grown in 250 ml of DMEM plus 10% serum in a 2-liter roller bottle. The medium was allowed to bind to the indicated antibody-coupled resin. The bound proteins were eluted and analyzed by immunoblotting. 10 ng of shHAI-1B (upper panel) or 25 ng of purified soluble prostasin (lower panel) was loaded in the last lanes. IgG, mouse IgG (control); 6E9, anti-HAI-1B monoclonal antibody.
HAI-1B Is an Inhibitor of Prostasin

FIGURE 4. Interactions of prostasin with HAI-1B in CHO/HAI-1B stable cell culture. A, HAI-1B is expressed on the cell membrane and spontaneously shed. Confluent CHO cells transfected with HAI-1B (CHO/HAI-1B) or vector alone (CHO/vector) were incubated in serum-free medium for 2 and 24 h. Both the cell extract and medium were subjected to immunoblotting analysis using anti-HAI-1B polyclonal antibody. CHO/HAI-1B or CHO/vector cells were incubated with or without 200 ng/ml purified soluble prostasin in serum-free medium for 2 h. The prostasin/HAI-1B complexes were co-immunoprecipitated from the medium (B and C) or the cell lysate (D). The last two lanes in D are 25 ng of sHAI-1B and 25 ng of purified soluble prostasin, respectively. E, CHO/HAI-1B or CHO/vector cells were transiently transfected with full-length prostasin, which was expressed on the cell membrane (lanes 1 and 2). The prostasin/HAI-1B complexes were co-immunoprecipitated from the cell membrane extract (lanes 3 and 4) and the medium (lanes 5 and 6) with anti-HAI-1B antibody 6E9, and detected by anti-prostasin antibody. IP, immunoprecipitation; IB, immunoblotting; IgG, mouse IgG (control); 6E9, anti-HAI-1B monoclonal antibody; PSS, anti-prostasin polyclonal antibody; PH, anti-HAI-1B polyclonal antibody.

DISCUSSION

In this study we used a bioinformatics approach, comparing gene expression profiles of prostasin with genes encoding protease inhibitors to identify physiologic regulators of prostasin. The highest scoring protease inhibitor found was the bi-Kunitz inhibitor HAI-1/1B. Reverse transcription-PCR experiments further demonstrated that prostasin expression correlated with the expression of both isoforms, HAI-1 and HAI-1B. In agreement, immunostaining studies showed that prostasin and HAI-1/1B proteins co-localize to the epithelium of prostate and ovarian tissues (6, 7, 12, 34). Like prostasin, HAI-1/1B is specifically expressed on the epithelial cell surface, from where it can be released in a soluble form. HAI-1/1B is known to inhibit several trypsin-like serine proteases, including the epithelial type II transmembrane serine protease matriptase (21). There is strong experimental evidence that matriptase and HAI-1/1B constitute a physiologic enzyme-inhibitor pair, including the isolation of matriptase/HAI-1 complexes from human breast milk (21). Therefore, we expected HAI-1/1B to appear as a matching protease inhibitor for matriptase in a co-expression analysis similar to that carried out for prostasin. Indeed, we found HAI-1/1B as the highest ranking protease inhibitor in seven tissues and as the second highest in two tissues (data not shown), thus corroborating the validity of our bioinformatics approach. The prostasin/HAI-1/1B pair had a similar overall score, HAI-1/1B ranking highest in seven tissues and second highest in six tissues. Consistent with these findings, a co-expression search starting with the HAI-1 probe set 202826 at revealed that prostasin and matriptase ranked as the highest and second highest serine proteases, respectively (data not shown).

Biochemical evidence supporting the proposal that HAI-1/1B and prostasin constitute an inhibitor-enzyme pair was derived from binding and inhibition studies using purified proteins and from cell culture systems. First, we showed that sHAI-1B formed a stable 1:1 complex with prostasin, indicating that only one of the two sHAI-1B Kunitz domains participated in prostasin binding. By using two sHAI-1B mutants in which the functions of KD1 and KD2 were separately inactivated by P1-directed mutagenesis, we were able to determine that KD1 accounted for essentially all of the binding activity. The results from prostasin enzymatic assays were entirely consistent with this conclusion, demonstrating that the inhibitory activity of KD2 was <6% of KD1. Therefore, the interaction of prostasin with HAI-1B via KD1 follows the same paradigm known for other target enzymes of HAI-1/1B, such as HGFA, matriptase, and hepsin (13, 19, 22, 23). It is unclear why KD2, which has ~75% sequence identity with KD1, largely lacks binding affinity to these proteases. It is conceivable that an unusual Glu at residue 402 in KD2 instead of a much smaller Gly or Ala found in KD1 and other Kunitz domain inhibitors might cause a steric conflict with its target enzymes, resulting in poor inhibitory activity (29). KD1 is identical in both splice variants, and therefore it was not surprising that the other isoform, sHAI-1, inhibited prostasin with a potency comparable with that of sHAI-1B. Furthermore, recombinant KD1 alone (Thr246_Val303) was a significantly more potent prostasin inhibitor than sHAI-1B and sHAI-1, which is in keeping with the 3–30-fold greater potency of recombinant KD1 for other proteases (29). This could mean that the protease interaction of KD1 is under the allosteric influences of other HAI-1/1B domains, perhaps of the region located C-terminal to KD1 comprising the low density lipoprotein (LDL) receptor-like and KD2 domains. Some support for this hypothesis comes from the observation that the shed 40-kDa fragment of HAI-1, which spans the N-terminal region ending with KD1, has greater binding affinity toward HGFA as compared with the 58-kDa fragment containing the additional LDL receptor-like and KD2 domains (35).

Studies with HAI-1/1B-expressing cell lines provided further evidence that prostasin and HAI-1/1B may interact in vivo. We found that similar to HGFA and matriptase (20, 21, 36), prostasin bound to HAI-1B on the cell surface and shed HAI-1/1B fragments as well. The experiments with OVCA3 cells, which endogenously express prostasin and both HAI-1 isoforms, demonstrated that complexes of prostasin and HAI-1/1B were present in the conditioned medium. Because co-localization on the cell surface increases reactant concentrations and the probability of forming enzyme-inhibitor complexes, we consider it likely that the complexes were initially formed on the cell surface before being shed into the medium. Results from additional studies with a prostasin/HAI-1B co-expression system are consistent with this interpretation, demonstrating that prostasin/HAI-1B complexes were present on the CHO membrane surface as well as in the culture medium.
HAI-1B Is an Inhibitor of Prostasin

and, thus, could serve as a useful tool for further investigation of the role of prostasin enzymatic activity in tumor progression and metastasis.

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REFERENCES

1. Yu, J. X., Chao, L., and Chao, J. (1994) J. Biol. Chem. 269, 18843–18848
2. Chen, L. M., Skinner, M. L., Kaufman, S. W., Chao, J., Chao, L., Thaler, C. D., and Chai, K. X. (2001) J. Biol. Chem. 276, 21434–21442
3. Yu, J. X., Chao, L., and Chao, J. (1995) J. Biol. Chem. 270, 13483–13489
4. Donaldson, S. H., Hirsh, A., Li, D. C., Holloway, G., Chao, J., Boucher, R. C., and Gabriel, S. E. (2002) J. Biol. Chem. 277, 8338–8345
5. Tong, Z., Illek, B., Bhagwandin, V. J., Verghese, G. M., and Caughey, G. H. (2004) Am. J. Physiol. 287, 1928–1935
6. Mok, S. C., Chao, J., Skates, S., Wong, K., Yiu, G. K., Muto, M. G., Berkowitz, R. S., and Cramer, D. W. (2001) J. Natl. Cancer Inst. 93, 1458–1464
7. Chen, L. M., Hodge, G. B., Guarda, L. A., Welch, J. L., Greenberg, N. M., and Chai, K. X. (2001) Prostate 48, 93–103
8. Takahashi, S., Suzuki, S., Inaguma, S., Ikeda, Y., Cho, Y. M., Hayashi, N., Inoue, T., Sugimura, N., Yishiya, N., Fujita, T., Chao, J., Ushijima, T., and Shirai, T. (2003) Prostate 54, 187–193
9. Chen, L. M., and Chai, K. X. (2002) Int. J. Cancer 97, 323–329
10. Chen, L. M., Zhang, X., and Chai, K. X. (2004) Prostate 59, 1–12
11. Bouton, M. C., Richard, B., Rossignol, P., Philippe, M., Guillin, M. C., Michel, J. B., and Jandrot-Perrus, M. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 142–147
12. Katoaka, H., Suganuma, T., Shimomura, T., Itoh, H., Kitamura, N., Nabeshima, K., and Kono, M. (1999) J. Histochem. Cytochem. 47, 673–682
13. Kirchhofer, D., Peek, M., Li, W., Stamos, J., Eigenbrod, C., Kadicchyan, S., Elliott, J. M., Corpuz, R., Lazarus, R. A., and Moran, P. (2003) J. Biol. Chem. 278, 36341–36349
14. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) J. Biol. Chem. 272, 6370–6376
15. Tanaka, H., Nagaike, K., Takeda, N., Itoh, H., Kohama, K., Fukushima, T., Miyata, S., Uchiyama, S., Uchinokura, S., Shimomura, T., Miyazawa, K., Kitamura, N., Yamada, G., and Katoaka, H. (2005) Mol. Cell. Biol. 25, 5687–5698
16. Itoh, H., Katoaka, H., Tomita, M., Hamasuna, R., Nawa, Y., Kitamura, N., and Kono, M. (2000) Am. J. Physiol. 278, G635–G643
17. Katoaka, H., Uchino, H., Denda, K., Kitamura, N., Itoh, H., Tsuobuchi, H., Nabeshima, K., and Kono, M. (1998) Cancer Lett. 128, 219–227
18. Katoaka, H., Hamasuna, R., Itoh, H., Kitamura, N., and Kono, M. (2000) Cancer Res. 60, 6148–6159
19. Denda, K., Shimomura, T., Kawaguchi, T., Miyazawa, K., and Kitamura, N. (2002) J. Biol. Chem. 277, 14053–14059
20. Katoaka, H., Shimomura, T., Kawaguchi, T., Hamasuna, R., Itoh, H., Kitamura, N., Miyazawa, K., and Kono, M. (2000) J. Biol. Chem. 275, 40453–40462
21. Lin, C. Y., Anders, J., Johnson, M., and Dickson, R. B. (1999) J. Biol. Chem. 274, 18237–18242
22. Kirchhofer, D., Peek, M., Lipari, M. T., Billeci, K., Fan, B., and Moran, P. (2005) FEBs Lett. 579, 1945–1950
23. Herter, S., Piper, D. E., Aaron, W., Gabriele, T., Cutler, G., Cao, P., Bhattacharjee, A., Choe, T., and Siani-Rose, M. A. (2003) J. Biol. Chem. 278, 5790–5797
24. Lee, S. I., Dickson, R. B., and Lin, C. Y. (2000) J. Biol. Chem. 275, 36720–36725
25. Takesuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R., and Caira, C. S. (2000) J. Biol. Chem. 275, 26333–26342
26. Muthukumar, T., Ding, R., Daddah, D., Medeiros, M., Li, B., Sharma, V. K., Hartono, C., Serur, D., Seshan, S. V., Volki, H. D., Beineke, K., and Suthanthiran, M. (2003) Transplantation 75, 1565–1570
27. Phillips, T., Opperman, J. T., Shah, R., Liu, N., Froelich, C. J., and Ashton-Rickardt, P. G. (2004) J. Immunol. 173, 3801–3809
28. Tyagi, S. C., Kumar, S. G., Banks, J., and Fortson, W. (1995) J. Mol. Cell. Cardiol. 27, 2177–2189
29. Saha, S., Samyoth, K., Kirchhofer, D., Fan, B., Wu, J., Corpuz, R. T., Santell, L., Lazarus, R. A., and Eigenbrod, C. (2005) J. Mol. Biol. 346, 1335–1349
30. Dennis, M. S., Herzka, A., and Lazarus, R. A. (1995) J. Biol. Chem. 270, 25411–25417
31. Saken, T. G., and Eisenberg, D. (2001) Nat. Genet. 29, 295–300
32. Liu, G., Lorraine, A. E., Shigeta, R., Cline, M., Cheng, J., Valmeekam, V., Sun, S., Kulp, D., and Siani-Rose, M. A. (2003) Nucleic Acids Res. 31, 82–86
HAI-1B Is an Inhibitor of Prostasin

33. Kohler, G., and Milstein, C. (1975) Nature 256, 495–497
34. Oberst, M. D., Johnson, M. D., Dickson, R. B., Lin, C. Y., Singh, B., Stewart, M., Williams, A., al-Nafussi, A., Smyth, J. F., Gabra, H., and Sellar, G. C. (2002) Clin Cancer Res. 8, 1101–1107
35. Shimomura, T., Denda, K., Kawaguchi, T., Matsumoto, K., Miyazawa, K., and Kitamura, N. (1999) J. Biochem. (Tokyo) 126, 821–828
36. Benaud, C., Oberst, M., Hobson, J. P., Spiegel, S., Dickson, R. B., and Lin, C. Y. (2002) J. Biol. Chem. 277, 10539–10546
37. Lin, C. Y., Wang, J. K., Torri, J., Dou, L., Sang, Q. A., and Dickson, R. B. (1997) J. Biol. Chem. 272, 9147–9152
38. Marlor, C. W., Delaria, K. A., Davis, G., Muller, D. K., Greve, J. M., and Tamburini, P. P. (1997) J. Biol. Chem. 272, 12202–12208
39. Shipway, A., Danahay, H., Williams, J. A., Tully, D. C., Backes, B. J., and Harris, J. L. (2004) Biochem. Biophys. Res. Commun. 324, 953–963
40. Kawaguchi, T., Qin, L., Shimomura, T., Kondo, J., Matsumoto, K., Denda, K., and Kitamura, N. (1997) J. Biol. Chem. 272, 27558–27564
41. Parr, C., and Jiang, W. G. (2001) Int. J. Oncol. 19, 857–863
42. Klezovitch, O., Chevillet, J., Mirosevich, J., Roberts, R. L., Matusik, R. J., and Vasioukhin, V. (2004) Cancer Cell 6, 185–195
43. Miyazawa, K., Shimomura, T., and Kitamura, N. (1996) J. Biol. Chem. 271, 3615–3618
