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Inhibitors of prostatin

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INHIBITORS OF PROSTASIN

The National Cancer Institute.

Chen (Int J Cancer 97(3), 323-329, 2002).*

Donaldson (J. Biol. Chem. 277, 8338-8345, 2002).*


(Continued)

Primary Examiner—David Lukton
(74) Attorney, Agent, or Firm—Allen, Dyer, Doppelt, Milbrath & Gilchrist, P.A.

ABSTRACT

A class of proteins useful as inhibitors of prostatase and method for identifying them are provided. These proteins have the structure wherein the amino acids P1-P4 from the scissile bond are respectively leu-ile-ala-arg and the amino acids at positions P5-P15 are serpin sequences.

4 Claims, 7 Drawing Sheets


* cited by examiner
Figure 1

SEQ ID No. 3

<table>
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<tr>
<th>G</th>
<th>B</th>
<th>X</th>
<th>Z</th>
<th>Z</th>
<th>Z</th>
<th>Z</th>
<th>X</th>
<th>X</th>
<th>X</th>
<th>X</th>
<th>L</th>
<th>I</th>
<th>A</th>
<th>R</th>
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</thead>
<tbody>
<tr>
<td>P15</td>
<td>P14</td>
<td>P13</td>
<td>P12</td>
<td>P11</td>
<td>P10</td>
<td>P9</td>
<td>P8</td>
<td>P7</td>
<td>P6</td>
<td>P5</td>
<td>P4</td>
<td>P3</td>
<td>P2</td>
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</table>

Figure 2A

SEQ ID No. 1

<table>
<thead>
<tr>
<th>L</th>
<th>I</th>
<th>A</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>P3</td>
<td>P2</td>
<td>P1</td>
</tr>
</tbody>
</table>

L = Leu
I = Ile
A = Ala
R = Arg

Figure 2B

SEQ ID No. 2

<table>
<thead>
<tr>
<th>G</th>
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<th>Z</th>
<th>Z</th>
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<td>P9</td>
<td>P8</td>
<td>P7</td>
<td>P6</td>
<td>P5</td>
</tr>
</tbody>
</table>

X = Any amino acid
Z = short chain amino acids such as Ala, Gly or Ser
B = Thr or Ser
G = Gly
Figure 3
Figure 6
Figure 7
Mouse |
--- |
118 K | Complex |
82 K | GST-PN-1 |
50 K | Prostasin |
33 K | |
27 K | |

Human |
--- |

1 2 3 4 5 6

Figure 8
INHIBITORS OF PROSTASIN

The present invention relates to identification of inhibitors of prostasin, and claims the benefit of priority from Provisional Patent Application 60/379,469 filed on May 10, 2002.

Funded in part by the Department of Defense Prostate Cancer Research Program Grants Number DAMD 17-98-1-8590, and DAMD 17-02-1-0032.

FIELD OF THE INVENTION

Background of the Invention

Prostasin is a serine protease known to be expressed at highest levels in the semen and prostate, however, it is also expressed in other tissues at low levels. In seminal fluid prostasin can be found in complex with a prostasin binding protein that can inhibit prostasin enzymatic activity. Prostasin mRNA is found in normal prostate epithelial cells but is not expressed in invasive prostate cancer lines. Expression of prostasin in invasive cancer lines reduces the invasiveness of cells in vitro. Prostasin is a serine protease that may have roles in normal prostate function and in suppression of tumor cell invasion.

Prostasin expression has also been implicated in ovarian and breast cancers. It is down-regulated in invasive breast cancer cell lines.

Prostasin is also known to be an activator of the epithelial sodium channel in vitro, and is present in tissues that absorb Na+ such as the kidney, colon, lung, and salivary glands. The proper regulation of the epithelial sodium channel is crucial to maintaining sodium balance, extracellular fluid volume and blood pressure. As such, it is a protein whose regulation and expression are implicated in diseases of the kidney, hypertension, and respiratory diseases.

Prostasin may also have a role in regulating various aspects of the male reproductive system, which affect male fertility.

Prostasin’s physiological functions are not well characterized, and at present, neither are its substrates, inhibitors, co-factors or other regulators. Identifying these factors would be very advantageous in the ultimate development of drug therapies, assays and diagnostic tools for diseases that relate to the regulation and function of prostasin.

Prostasin belongs to the group of proteases called “serine proteases.” Serine proteases are a family of enzymes that cut peptide bonds in other proteins. This activity is dependent on a set of amino acid residues in the active site of the enzyme, one of which is always a serine.

Serine proteases are inhibited by a group of inhibitors named serpins, so called because they are serine protease inhibitors. Structurally, serpins have a central sheet and an exposed reactive center loop (RCL) at the top of the protein containing the target cleavage sequences. Serpins work by mimicking the three-dimensional structure of a normal substrate of the protease, and the serine protease binds the serpin instead of its normal substrate. This function alone would block any further activity by the proteases. However, the proteases also cleave the serpin, forming a covalent bond linking the molecules and making a change in the three dimensional structure of the serpin which moves the attached protease to a location where it can be destroyed.

U.S. Pat. No. 6,420,157 to Durnow, et al. describes an expression vector system which identifies modulators and substrates of prostasin, but the present invention permits identification of such substances without laboratory experimentation.

SUMMARY OF THE INVENTION

One aspect of the invention relates to inhibitors of prostasin.

Another aspect of the invention relates to a method for inhibiting prostasin.

Another aspect of the invention relates to a method for identification of amino acid sequences that are the best inhibitors of prostasin.

Another aspect of the invention relates to methods for selecting compounds which are therapeutically useful for treating diseases which involve prostasin regulation.

A further aspect of the invention relates to methods for selecting compounds which are useful for regulation of male fertility.

Further objects and advantages of this invention will be apparent from the following detailed description of a presently preferred embodiment which is illustrated schematically in the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the amino acid sequence for the “leu-ile-alaa-arg (LIAR)” sequence of the invention (SEQ ID No. 3).

FIG. 2 shows the amino acid sequence for a “regular” serpin sequence (SEQ ID No 2).

FIG. 3A shows representative purification profiles for each purification step.

FIG. 3B shows proteins from each purification step subjected to SDS-PAGE/Coomassie blue staining.

FIG. 3C shows a Western blot analysis using mPBP antibody.

FIG. 4A shows a Western blot analysis using mPBP antibody and a prostasin antibody and both antibodies recognize prostasin-mPBP complex at 82 kDa. Prostasin antibody recognizes purified prostasin but not purified mPBP. The mPBP antibody recognizes purified mPBP but not purified prostasin.

FIG. 4B shows the time course of complex formation inhibited between prostasin and mPBP.

FIG. 4C shows mPBP—prostasin complex formation inhibited in presence of serine protease inhibitor aprotinin (lane 3) PMSF (lane 4) or heparin (lane 5) or mPBP antibody (lane 1) lane 2 is prostasin-mPBP complex as a control.

FIG. 5 shows purified mPBP trypsin digested and separated using Tricine/SDS-PAGE, and immunodetected with mPBP antibody. Band indicated with arrow sent for sequence analysis and shown to be identical to sequences between position 26 and 57 of PN-1.

FIG. 6A shows prostasin-mPBP complex with thrombin and thrombin-mPBP formation and its pH dependence.

FIG. 6B shows Heparin at 0.25 unit/reaction abolishes complex formation between prostasin-mPBP but not between thrombin-mPBP.

FIG. 7 shows inhibition of prostasin activity when incubated with mPBP at different concentrations.

FIG. 8 shows GST-mPN-1 (lane 1) and GST-hPN-1 (lane 4) form a 100 kDa complex when incubated with prostasin (lanes 2 and 5). Complex formation is inhibited by aprotinin (lanes 3 and 6).

DETAILED DESCRIPTION OF THE INVENTION

Before explaining the disclosed embodiment of the present invention in detail it is to be understood that the invention is not limited in its application to the details of the particular arrangement shown since the invention is capable of other
Amino terminus \ldots P_{15} \ldots P_{2}P_{3}P_{4} \ldots Carboxyl

In classical "lock and key" theory of enzyme-substrate action, the protease is the "lock" having openings that precisely match the "key" or substrate that comes in to fit the protease. An optimally fit substrate is the best substrate and will be cleaved at the scissile bond. The fate of the protease substrate interaction is largely dependent on the types of residues at \( P_{2}-P_{15} \) which determine that it is either a substrate, or an inhibitor.

Once a substrate binds to the prostasin protease via its \( P_{1}-P_{4}a \) cleavage will occur at the scissile bond, and residues to the right of the cleavage (i.e., \( P_{1}-P_{4}a \) and beyond) will dissociate from the protease. If \( P_{5}-P_{15} \) are random and not a "regular" serpin sequence. A "regular" serpin sequence is one in which a consensus has determined that \( P_{2}-P_{12} \) are occupied by residues with short side-chains such as alanine, glycine or serine, and P14 is usually threonine or serine and \( P_{15} \) is usually glycine. The regular serpin sequence is hereinafter referred to as Sequence ID 2 and shown in FIG. 2.

In classical "lock and key" theory of enzyme-substrate action, the protease is the "lock" having openings that precisely match the "key" or substrate that comes in to fit the protease. An optimally fit substrate is the best substrate and will be cleaved at the scissile bond. The fate of the protease substrate interaction is largely dependent on the types of residues at \( P_{2}-P_{15} \) which determine that it is either a substrate, or an inhibitor.

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ther peak c (the flow-through) nor peak d contained any detectable amount of mPBP. The mPBP was detected in peak e (indicated by the horizontal solid bar). The flow through fractions (peak f) did not contain mPBP. The purified mPBP was used to generate a polyconal antibody using rabbit as the host. (see example 2 below) Proteins from each purification step were subjected to SDS PAGE/Coomassie blue staining (FIG. 3B) or western blot analysis using the mPBP antibody (FIG. 3C). As shown in FIG. 3B, the purified mPBP (lane 4, 5 micrograms) migrated at 45 kDa in an SDS-PAGE under reducing conditions. As shown in FIG. 3C, the mPBP antibody recognized a 45 kDa protein in mouse seminal vesicle fluid, as well as the purified mPBP itself.

Example 2

Identification of mPBP as Protein Nexin-1

Preparation of Polyclonal Antiserum

An anti-serum against PBP was prepared according to the procedure described in Chen et al. Briefly, 0.5 ml of the purified mPBP (250 micrograms) was emulsified with an equal volume of complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, Mo.) and injected subcutaneously into a 1.5 kg New Zealand White female rabbit (Charles River Laboratories, Wilmington, Mass.). Booster injections were made with 100 micrograms of mPBP (emulsified with incomplete Freund’s adjuvant, Sigma-Aldrich) for 3 times at 3-week intervals. Pre-immune rabbit serum was collected before the initial immunization.

Prostatin-Binding Assay and Western Blot Analysis

The procedures were performed according to Chen et al. Briefly, purified recombinant prostatin was incubated with samples from each purification step or the final purified mPBP at 37 degrees C. for 1 hour or for various times as indicated. The binding reaction was stopped by the addition of SDS sample buffer (1x SDS sample buffer=62.5 mM Tris-Hcl at pH 6.8, 2% (v/v) glycerol, 2% SDS (w/v) and 2% Beta-mercaptoethanol). The reaction mixtures were then boiled for 5 min, and resolved in 10% SDS-polyacylamide gels. The resolved proteins were then transferred to nitrocellulose membranes, and analyzed with either a prostatin antibody or the mPBP antibody. Signals were detected using an ECL detection procedure with the WestPico reagents (Pierce, Rockford, Ill.) following the manufacturer’s protocol. The membrane was then exposed to X-ray film (Midwest Scientific, St. Louis, Mo.) The prostatin antibody was used at 1:2,000 dilution, the mPBP antibody was used at 1:10,000 dilution, and the secondary antibody (goat anti-rabbit IgG, Sigma-Aldrich) was used at 1:10,000 dilution. All antibodies were diluted in 5% non-fat milk in TBS-T (TBS-T=20 mM Tris-Hcl at pH 7.6, 0.14M NaCl and 0.1% Tween-20).

Amino Acid Sequence Analysis

The purified mPBP (6 micrograms) was incubated with various amounts of trypsin (Life Technologies) at 37 degrees C. for 30 minutes, and subjected to Tricine/SDS-PAGE (Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368-379) followed by transferring to the Immobilon-P membrane (Fisher, Pittsburgh, Pa.). One membrane was subjected to immunodetection with the mPBP antibody, an identical membrane was stained with 0.02% Coomassie blue R-250 in 40% methanol and 5% acetic acid for 30 seconds. The membrane was then destained in 40% methanol and 5% acetic acid for 1 min, rinsed in distilled water for 3x5 min to remove the destaining solution, and air dried. A stained band at ~10 kDa, which was recognized by the mPBP antibody, was for amino acid sequence analysis at the Protein Core Facility of the University of Florida (Gainesville, Fla.).

Enzymatic Assay

Recombinant human prostatin was purified as described in Chen et al. A synthetic substrate, N-t-Boc-Glu-Ala-Ala-Arg-7-amido-4-methyl coumarin (QAR-AMC) was purchased from Sigma-Aldrich. The purified mPBP (concentration range 0.4 micromolar) was incubated with prostatin (0.8 micromolar) for 30 mins at 37 degrees C. The binding reaction mixture (20 microliters) was then added to 80 microliters of 50 micromolar Tris-HCl pH 8.0/0.1% bovine serum albumin containing the QAR-AMC substrate (final concentration: 100 micromolar) in 96-well microtiter plates (Costar, Cambridge, Mass.). The velocity of substrate hydrolysis was measured using a Wallac 1420 Victor multilabel counter at wavelength 355 nm and wavelength 460 nm. The residual activity of prostatin (velocity of the inhibited enzyme reaction/velocity of the uninhibited enzyme reaction) as plotted versus the mPBP concentration.

Molecular Cloning, Expression, and Purification of Recombinant Mouse and Human Protease Nexin-1 (PN-1, Spi-4)

A cDNA encoding the mature peptide of mouse protease nexin-1 (PN-1 or Spi-4) was cloned from mouse seminal vesicle mRNA by reverse-transcription-polymerase chain reaction (RT-PCR). Total RNA of mouse seminal vesicle was isolated using a procedure described in Chirgwin, J. M., Przybyla, A. E., MacDonal, R. J. and Rutter, W. J. (1979) Biochemistry 18:5294-5299 (incorporated herein by reference), an Oligotex mRNA Mini Kit from QIAGEN (Valencia, Calif.) was used to isolate the mRNA. The following oligonucleotide primers were used in the RT-PCR to generate the mouse PN-1cDNA: upstream: 5’GGAGAATTC TCC CAG TTC GTT CAC CTG GC-3’ the downstream primer sequence corresponds to base numbers 206-225 of X70296, the first codon (206-208) is that of Ser, the amino-terminal residue of the mature mouse PN-1 peptide (Vassalli, 1993) The upstream primer sequence corresponds to base numbers 206-225 of X70296, the first codon (206-208) is that of Ser, the amino-terminal residue of the mature mouse PN-1 peptide (Vassalli, 1993) The downstream primer sequence corresponds to base numbers 1323-1342 including the termination Odon (1,340-1,342). The RT-PCR was performed as described in Chen, L. M., Hodge, G. B., Guardia, L. A., Welch, J. L., Greenberg, N. M. and Chai, K. X. (2001), and incorporated herein by reference using 3 micrograms of mouse seminal vesicle mRNA as the template. A single cDNA band was amplified. The Taq DNA polymerase was removed by phenol/chloroform extraction, and the cDNA was treated with EcoR I and Xho I under proper buffer conditions. The restriction-modified cDNA was then inserted into the p’GEX-6P-1 vector (Amersham Pharmacia Biotech) at the corresponding sites, resulting in a fusion gene construct that encodes GST-mPN-1 (GST: glutathione-S-transferase) The amplified PN-1 portion of the fusion gene was com-
Protein Binding Protein Inhibits Prostasin’s Activity

The protein-binding protein that was identified in the seminal vesicle inhibits prostatic activity as determined by membrane-overlay zymography. QuAR-AMC was used as a substrate for prostatin to test the inhibitory activity of the purified mpBP. Prostatin (0.8 micromolar) was incubated with 0, 0.05, 0.1, 0.2 or 0.4 micromolar mpBP at 37 degrees C for 30 mins. The reaction mixture was then to the assay buffer containing a final concentration of 100 micromolar QuAR-AMC substrate. As shown in Figure 7, when incubated with mpBP at different concentrations, prostatin’s activity was inhibited in a dose dependent manner.

Prostatin Forms a Complex with Recombinant protease nexin-1

To establish if mpBP is indeed protease nexin-1, mouse and human protease nexin-1 (PN-1) cDNA were cloned into the pGEX-6P-1 expression vector. The recombinant protein products have the schistosomal glutathione-5-transferase (GST) fused to the N-terminus of the PN-1. The GST fusion proteins were affinity-purified using glutathione-conjugated agarose-beads. For each type of recombinant protein, cleared supernatant of cell lysate from one liter of culture was incubated with 1 ml of 505 glutathione-beads. The beads were eluted with 1 ml of fresh 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Twenty microliters of the eluent were incubated with 0.5 micrograms of recombinant prostatin at 37 degrees C for 60 minutes in the absence or presence of aprotinin. Both the GST-mpBP-1 (Figure 8, lane 1 alone at 64 kDa) and the GST-hPN-1 (Figure 8, lanes 2 and 5). The complex formation was inhibited by the serine protease inhibitor aprotinin (Figure 8, lanes 3 and 6). These findings further indicate that the mpBP is the serpin protease nexin-1. For this immunoblot, both the protease antibody and the mpBP antibody were used as the primary antibody, and a goat anti-rabbit IgG conjugated with HRP was used as a secondary antibody. An immunoreactive band at 30 kDa and two other minor immunoreactive bands at 42 kDa and 60 kDa were likely the products of non-specific degradation of the recombinant PN-1, since no protease inhibitors were added in the cell lysate during purification.

Example 3

Identification of the reactive sequence of the serpin PN-1

The reactive site sequences of PN-1 at locations P₁-P₃ are known to be Leu-ile-Ala-Arg. It has been shown with the serpin Kallistatin that its reactive site sequence was also the best for a substrate.

Example 4

Heparin and Thrombin Binding to PN-1

It has been shown that PN-1 can form complexes with various serine proteases, including thrombin. Figure 6A shows the complex formation between prostatin-mPB and the

between position 26 and 37 of the GenBank mouse protease nexin-1 (PN-1) translated sequence (accession number X70196). This result suggests that PBP may be identical to PN-1, a serine protease inhibitor (serpin).

Prostatin Binding Protein Inhibits Prostasin’s Activity

The protein-binding protein that was identified in the seminal vesicle inhibits prostatic activity as determined by membrane-overlay zymography. QuAR-AMC was used as a substrate for prostatin to test the inhibitory activity of the purified mpBP. Prostatin (0.8 micromolar) was incubated with 0, 0.05, 0.1, 0.2 or 0.4 micromolar mpBP at 37 degrees C for 30 mins. The reaction mixture was then to the assay buffer containing a final concentration of 100 micromolar QuAR-AMC substrate. As shown in Figure 7, when incubated with mpBP at different concentrations, prostatin’s activity was inhibited in a dose dependent manner.

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Example 4

Heparin and Thrombin Binding to PN-1

It has been shown that PN-1 can form complexes with various serine proteases, including thrombin. Figure 6A shows the complex formation between prostatin-mPB and the
between thrombin-mPBP. We also tested if the complex is pH dependent because prostasin's optimum pH is 9.0. The results showed that the purified mPBP formed a complex with prostasin as well as thrombin. The complex formation of mPBP and prostasin is increased with higher pH in the binding conditions while the complex formation of mPBP and thrombin is somewhat decreased with increasing pH. We further tested if heparin may have a different effect on the complex formation between prostasin-mPBP versus that between thrombin-mPBP. In FIG. 6B, it is shown that heparin, at 0.25 unit/reaction, completely abolished the complex formation between prostasin—mPBP but not between thrombin-PMP.

A heparin binding site has been mapped in PN-1 by Stone, S. R. Brown-Luedi, M. L., Rovelli, G. Guidolin, A. McGlynn, E. and Monard, D. (1994) Biochemistry 33: 7731-7735. In the presence of heparin, the inhibitory activity of PN-1 to several serine proteases, such as thrombin and factor Xa is known to be enhanced. In the presence of heparin, however the binding between prostasin and PN-1 is abolished. Also, in an enzymatic assay using the QAR-AMC substrate, pre-incubation of mPBP and heparin was able to prevent prostasin inhibition by mPBP. This is a novel finding of PN-1’s serine protease inhibition mechanism, having potentially profound implications, especially in cancer biology. PN-1 can bind to heparin-like molecules, or heparan sulfate proteoglycans (HSPG) on the cell surface and this binding apparently accelerates thrombin inhibition by PN-1. The HSPG, as a component of the extracellular matrix (ECM) is suggested to play a major role in cell-matrix signaling. Since prostasin is a GPI-anchored membrane protease which has an anti-invasive activity in vitro, it is likely that prostasin’s anti-invasion activity is regulated by PN-1 and the ECM in the tissue microenvironment. Both the membrane bound and secreted prostasin may be a proteolytic regulator of cell surface events but may also serve as a receptor or a ligand in ECM signaling or tissue remodeling under physiological or pathological conditions.

The complex formation between PN-1 with two of its target enzymes, prostasin and thrombin, is affected by pH (FIG. 6). Changes in intracellular pH have been shown to be a mechanism of cell signaling. While the invention has been described, disclosed, illustrated and shown in various terms of certain embodiments or modifications which it has presumed in practice, the scope of the invention is not intended to be, nor should it be deemed to be, limited thereby and such other modifications or embodiments as may be suggested by the teachings herein are particularly reserved especially as they fall within the breadth and scope of the claims here appended.

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We claim:

1. A method of inhibiting prostasin, the method comprising administering to a subject in need thereof a serine protease inhibitor having a reactive center loop consisting of a peptide of the formula \( P_1 P_2 P_3 P_4 P_5 P_6 P_7 P_8 P_9 P_{10} P_{11} P_{12} P_{13} P_{14} P_2 P_1 \) wherein \( P_1 \) to \( P_{15} \) are amino acid residues, wherein the tetrapeptide subsequence \( P_2 - P_1 \) consists of SEQ ID NO:1, and wherein the subsequence \( P_{15} P_{14} P_{13} P_{12} P_{11} P_{10} P_9 P_8 P_7 P_6 P_5 \) consists of SEQ ID NO:2.

2. The method of claim 1, wherein the serine protease inhibitor consists of protease nexin-1 (PN-1).

3. A method of inhibiting prostasin, the method comprising contacting the prostasin with protease nexin-1 (PN-1).

4. A method for inhibiting prostasin, the method comprising administering to a subject in need thereof a serine protease inhibitor, wherein said serine protease inhibitor has a reactive center loop comprising SEQ ID NO:3.

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