An allosteric anti-hepsin antibody derived from a constrained phage display library

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The serine protease hepsin is highly upregulated in prostate cancer and is implicated in tumor progression. Therefore, specific inhibition of hepsin enzymatic activity by an antibody constitutes an attractive therapeutic approach. Here, we report the identification of the anti-hepsin antibody Fab25 by screening of a Fab phage display library with a restricted chemical diversity at the complementary determining regions. Hepsin with its S1 pocket occupied by 3,4-dichloro-isocoumarin was used as the ‘bait’ for library screening. Fab25 was highly specific and it potently inhibited hepsin activity toward a panel of synthetic and macro-molecular substrates. Biochemical and enzymatic studies with synthetic substrates of variable length suggested that Fab25 acts as an allosteric inhibitor based on non-competitive inhibition kinetics. Isothermal titration calorimetric experiments showed that the high-affinity (KD 6.1 nM) binding of Fab25 with hepsin is enthalpically driven. Despite an unusually long CDR-H3 loop with several potential hepsin cleavage sites (Lys, Arg residues), Fab25 was not processed by hepsin. Antibody-25 should be valuable for investigating hepsin’s role in cancer progression and for potential therapeutic applications. Furthermore, the herein presented phage display strategy using an active site-modified prototype should be widely applicable for identifying potential allosteric anti-protease antibodies.

Keywords: allostery/antibodies/hepsin/phage display/prostate cancer/serine protease

Introduction

Hepsin is a cell-surface-expressed type II transmembrane serine protease and was identified as one of the most highly upregulated genes in prostate cancer (Dhanasekaran et al., 2001). Hepsin protein expression was highest at sites of bone metastasis and in late-stage primary tumors (Xuan et al., 2006), suggesting a role in tumor progression. In accordance, in vivo studies demonstrated that overexpression of hepsin led to tumor progression and metastasis (Klevezitch et al., 2004; Li et al., 2009a). This may be related to hepsin-driven activation of pro-invasive pathways, as suggested by the ability of hepsin to activate pro-urokinase plasminogen activator (pro-uPA) (Moran et al., 2006), pro-hepatocyte growth factor (pro-HGF) (Herter et al., 2005; Kirchhofer et al., 2005), pro-macrophage stimulating protein (MSP) (Ganesan et al., 2011) and to cleave the basement membrane protein laminin-332 (Tripathi et al., 2008).

Therapeutic neutralization of hepsin could be of great interest for attenuating prostate cancer progression. However, there is currently a lack of specific and potent inhibitors. A PEGylated Kunitz domain inhibitor potently suppressed hepsin-driven invasive growth in vivo (Li et al., 2009a), but it lacked specificity, as it was the case for small organic hepsin inhibitors (Chevillet et al., 2008). Xuan et al. (2006) identified monoclonal anti-hepsin antibodies; however, they displayed substrate-specific inhibitory activity. Thus, there clearly is a need for hepsin inhibitors that are specific, potent and which completely block catalysis toward all physiologically relevant substrates.

Phage display of antibody Fab and scFv libraries was successfully applied to identify highly potent antibodies against trypsin-like serine proteases. Structural studies revealed how antibodies inhibited enzyme catalysis by allosteric or by direct substrate interference mechanisms (Wu et al., 2007; Farady et al., 2008; Ganesan et al., 2009). Unlike the naturally occurring canonical inhibitors, interaction with the protease S1 specificity pocket is not a requisite for antibodies to inhibit catalysis (Wu et al., 2007; Ganesan et al., 2009). Therefore, to obtain an inhibitory hepsin antibody, we utilized modified hepsin with its S1 pocket filled with a small mechanism-based inhibitor to screen a phage-displayed Fab library. This led to the identification of Fab25, which potently and specifically inhibited hepsin enzymatic activity by an allosteric inhibition mechanism. Based on its excellent biochemical properties, Ab25 may potentially aid in dissecting the role of hepsin in prostate cancer and could be of potential therapeutic use.

Materials and methods

Reagents and proteins

The synthetic para-nitroanilide (pNA) substrates S2765 (= DiaPharma FXa substrate), S2266, S2288, S2366, S2444 were from DiaPharma (Westchester, OH, USA), Spectrozyme fIXa® (#299) and Spectrozyme® FVIIa from American Diagnostica (Greenwich, CT, USA). The 3,4-dichloro-isocoumarin (DCI), bovine serum albumin and Tween-20 were from Sigma-Aldrich. Factor VII was purchased from Enzyme Research and pro-uPA from Fitzgerald Industries. Pro-MSP and pro-HGF were recombinantly expressed as described (Kirchhofer et al., 2005; Ganesan et al., 2011). Plasmin and...
factor Xla were from Hematologic Technologies Inc. (Essex Junction, VT, USA), plasma kallikrein from Calbiochem (La Jolla, CA, USA), Factor XIIIa from Enzyme Research (South Bend, IN, USA), uPA from American Diagnostica. Matriptase (MT-SP1), prostatin, marapsin, hepatocyte growth factor activator (HGFA) were recombinantly expressed and purified as described previously (Kirchhofer et al., 2003; Li et al., 2009b; Camerer et al., 2010). As control antibodies, we used the anti-HGFA Fab or IgG (with the same framework residues as Camerer et al., 2010) toward S2765 substrate and complete inhibition was achieved under these experimental conditions.

YSGX library (10 ng/ml of biotinylated hepsin: DCI complex was incubated with 1 ml of amplified phage at 4°C for 1.5 h with 100 μM DCI. Phage was captured for 15 min with MyOne Streptavidin and eluted with 0.1 M HCl, immediately neutralized and then amplified following the standard protocol (Sidhu et al., 2000). From the second round forward, 2 μg of biotinylated hepsin was incubated with 400 μl of amplified phage at 4°C for 1.5 h with 100 μM DCI. Phage was captured for 15 min on Maxisorp Immunoplates (NUNC) coated with NeutrAvidin or streptavidin (alternated between rounds) and blocked with PBS, 0.5% BSA. After five rounds, individual clones were screened following the standard protocol (Sidhu et al., 2000) using biotinylated hepsin immobilized on NeutrAvidin-coated 384-well Maxisorp Immunoplates in the presence of 100 μM DCI. The identified Fab25 was reformatted into IgG (Ab25) by cloning the Fab25 variable domain of light chain and heavy chain into a pRK5-based plasmid with human light chain or heavy chain (IgG1) constant domain. Ab25 was expressed in CHO cells and Fab25 in Escherichia coli and purified by Protein-A affinity chromatography.

**Fab25 specificity**

A panel of nine trypsin-like serine proteases was incubated with 1 μM Fab25 for 40 min in Heps buffer (20 mM Heps, pH 7.5, 150 mM NaCl, 5 mM CaCl2, 0.01% Triton X-100), chromogenic substrates were added and increase in absorbance at 405 nm measured. The reactant concentrations were: 1 nM hepsin—0.5 mM S2765, 0.5 mM matriptase—0.5 mM S2765, 5 nM prostatin—0.5 mM S2765, 2 nM plasmin—0.5 mM S2366, 2 nM plasma kallikrein—0.5 mM S2366, 0.5 nM Factor Xla—0.5 mM S2366, 5 nM FXIIa—0.5 mM S2288, 5 nM uPA—0.5 mM S2444, 50 nM marapsin—0.2 mM Spectrozyme® FVIIa, 10 nM HGFA—0.2 mM Spectrozyme® FVIIa. Data were expressed as fractional enzyme activity (v/vo) (n = 3–4 ± SD).

**In vitro proteolytic processing of Fab25 by hepsin**

Fab25 (2 μM) was incubated with 100 nM hepsin for 24 h at room temperature, either in a low-pH buffer (100 mM MES pH 6.0, 150 mM NaCl) or in a high pH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl). The reaction was stopped by the addition of 20 μl of 2X-sample buffer (with/without β-mercaptoethanol) and heated at 95°C for 5 min. Proteolysis was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 4–20% (w/v) gradient gel stained with SimplyBlue™.

**Enzyme assays**

Fab25, Ab25, control Fab or control IgG were incubated with hepsin (1 nM for human and 2 nM for mouse hepsin) in Heps buffer for 40 min. S2765 was added (0.2 mM) and the linear rates of the increase in absorbance at 405 nm measured on a kinetic microplate reader (SpectraMax M5, Molecular Devices). Data were expressed as fractional activity (v/vo) and fitted to a four-parameter fit to determine the IC50 values.

For enzyme kinetic experiments hepsin (1 nM) was incubated with Fab25 (9–1.2 nM) in Heps buffer for 40 min. S2765 (1–0.039 mM) was added and the linear rates of absorbance increase measured. Data were plotted as Eadie–Hofstee plots. For experiments with Bz-Arg-pNA and Z-Lys-Arg-pNA (Bachem, Torrance, CA, USA), hepsin (20 nM) was incubated with Fab25 (0, 10 and 25 nM) in Heps buffer for 30 min before substrate addition and initial velocities were measured. Vmax and Km values were determined from Michaelis–Menten plots.

**Macromolecular substrate assays**

Hepsin (50 nM) was incubated with 500 nM Fab25 or control Fab for 5 min in Heps buffer and 1.25 μM pro-HGF or pro-MSP or 8 μM factor VII were added. After 30 min/37°C, aliquots were analyzed by SDS-PAGE (4–20% gradient gel), gels were stained with SimplyBlue™ (Invitrogen). Pro-uPA activation by hepsin was measured essentially as described (Li et al., 2009a). Fab25 was incubated with hepsin (0.5 nM) and pro-uPA (100 nM) for 35 min. Aliquots were withdrawn at different time points, the generated uPA quantified with S2444 and initial velocities calculated. Cellular pro-HGF activation was measured by using hepsin-overexpressing LnCaP-34 cells as described (Moran et al., 2006).
Cell migration assay

Cell migration assays were performed essentially as described (Tripathi et al., 2008), using a 24-well, 8.0 μm pore size FluoroBlok™ permeable supports (BD Biosciences, Bedford, MA, USA). The underside of the filters were coated with either untreated or hepsin-treated rat laminin (1 μg/ml) in PBS overnight at 4°C as follows: (i) laminin alone, (ii) laminin + hepsin (10 nM), (iii) laminin + hepsin (10 nM) + Fab25 (100 nM). After blocking with superblock buffer for 1 h, DU145 cells (20 000/well) were seeded in the upper chamber in serum-free medium. After 5 h incubation in 5% CO2 and 37°C, migrated cells were fixed with 500 μl of Methanol for 30 min, air dried for 20 min and stained with 500 μl of 10 nM YO-PRO-I (Invitrogen, Carlsbad, CA, USA) for 10 min. After washing with PBS, fluorescence was measured in a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

Determination of equilibrium binding constants

Rabbit anti-human IgG were chemically immobilized (amine coupling) on CM5 biosensor chips and Ab25 was captured to give approximately 350 response units on a BLAcore™-3000 instrument (GE Healthcare, NJ, USA). An NHS/EDC-activated and de-activated surface was used as a control surface. Serial dilutions of active hepsin (1–500 nM) were injected in PBS overnight at 4°C as follows: (i) laminin alone, (ii) laminin + hepsin (10 nM), (iii) laminin + hepsin (10 nM) + Fab25 (100 nM). After blocking with superblock buffer for 1 h, DU145 cells (20 000/well) were seeded in the upper chamber in serum-free medium. After 5 h incubation in 5% CO2 and 37°C, migrated cells were fixed with 500 μl of Methanol for 30 min, air dried for 20 min and stained with 500 μl of 10 nM YO-PRO-I (Invitrogen, Carlsbad, CA, USA) for 10 min. After washing with PBS, fluorescence was measured in a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

Isothermal titration calorimetry (ITC)

The sample cell (204 μl) of an ITC200 instrument (GE healthcare) was loaded with the active hepsin (138 μM) obtained from the in vitro processing of a single-chain hepsin (with an engineered enterokinase cleavage site) by enterokinase and repurified by size-exclusion chromatography. About 10-fold molar excess of Fab25 (14 μM) was loaded in the syringe. A titration experiment typically consisted of 20 injections, each of 2 μl volume and 180 s duration, with 3 min intervals. Raw data were integrated, corrected for non-specific heats, normalized for concentration and analyzed according to a 1:1 binding model assuming a single set of identical binding sites. The data were integrated to provide a titration curve. By using a non-linear least-squares fit, the binding constant $K_A$, the heat of binding ($\Delta H$) and the stoichiometry of binding were extracted from the curve.

Results

Identification of Fab25 by antibody phage display with active-site-modified hepsin

DCI is a mechanism-based serine protease inhibitor that occupies the S1 pocket and completely inhibits hepsin activity at concentrations above 20 μM (Supplementary Fig. S1). Based on previous structural studies of antibody:protease interaction, S1 occupancy by DCI should not prevent antibody binding (Wu et al., 2007; Ganesan et al., 2009), but was expected to stabilize the active site, favorable for antibody interactions. Therefore, 100 μM DCI was included in phage sorting experiments using a minimalist synthetic antibody library designated as ‘YSGX library’ with chemical diversity at the complementarity determining regions (CDR) restricted to Tyrosine, Serine, Glycine and X (X represents equimolar mixture of all amino acids except for Cys, Tyr, Ser and Gly) at the molar ratio of 20 : 15 : 15 : 50. One clone (designated as Fab25) became dominant after five rounds of selection (CDR sequences in Fig. 1). For subsequent studies Fab25 and the reformatted IgG (Ab25) were expressed in E.coli and CHO cells, respectively, and purified.

Potency and specificity of Fab25

Fab25 completely inhibited human and mouse hepsin enzymatic activity with IC50 values of 4.1 ± 1.0 and 329 ± 51 nM, respectively, while a control Fab had no effect (Fig. 2A). A previously published anti-hepsin monoclonal antibody only partially blocked hepsin activity in enzymatic assays with the pNA substrate S2266 (Xuan et al., 2006). Therefore, we examined a panel of 7 pNA substrates all having a P1-Arg residue. Fab25 but not a control Fab inhibited hepsin-mediated cleavage of all pNA substrates by almost 100% including S2266 (Supplementary Table S1). Fab25 was highly specific for hepsin as shown by the lack of inhibitory activity toward nine trypsin-like serine proteases, including the closest homologues plasma kallikrein, prostanin, marapsin and plasmin (Fig. 2B).

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<td>GFNSYSSYM</td>
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Fig. 1. CDR sequences of Fab25. Depicted are the Fab25 CDR sequences (Kabat numbering) of light (L1, L2, L3) and heavy (H1, H2, H3) chains. Charged residues potentially involved in charge-charge interactions with hepsin are underlined.
Inhibition of macromolecular substrate processing by Fab25

Fab25 strongly inhibited the hepsin-mediated processing of zymogen factor VII, pro-HGF and pro-MSP by hepsin was carried out as indicated and monitored by SDS-PAGE. (B) Pro-uPA processing by hepsin. (C) 125I-labeled pro-HGF processing by hepsin-overexpressing LnCaP-34 cells. (D) Laminin-dependent migration of DU145 cells. DU145 cells (2 x 10⁵) in serum-free medium were added to pre-treated upper chambers of filter inserts and allowed to migrate for 5 h at 37°C. Cells that migrated to the bottom of the filters were fixed, stained with the Yo-Pro-1 and relative fluorescence units were measured. Hepsin-cleaved laminin stimulated DU154 cell migration, which was reduced to basal levels with Fab25.

Antibody binding is influenced by activation state of hepsin

Surface plasmon resonance (SPR) measurements showed that immobilized Ab25 bound to hepsin with a KD of 10.5 ± 0.2 nM (Fig. 4A). This result as well as the weaker binding affinity determined for mouse hepsin (KD 132.8 ± 17 nM) are in reasonably good agreement with the IC50 values from enzymatic assays (Fig. 2A). A zymogen-like single-chain form of hepsin with an engineered enterokinase cleavage site had no enzymatic activity, whereas treatment with enterokinase generated fully active hepsin (data not shown). Ab25 affinity to this single-chain form was ~1000-fold reduced (KD 5.52 ± 0.5 μM) (Fig. 4B), suggesting that Ab25 may
bind to a region, where major conformational differences between zymogen and active enzyme occur.

Thermodynamics of Fab25 binding to Hepsin measured by ITC showed that the binding reaction was exothermic and the stoichiometry was close to 1 : 1 (Fig. 4C). The calculated $K_D$ value was 6.13 nM, in excellent agreement with SPR measurements. The $D_H$ value of –27.53 kcal/mol indicated enthalpically driven binding, perhaps involving interactions of charged residues. The unfavorable entropy term ($-T\Delta S = +16.33$ kcal/mol) may suggest ordering of hepsin and/or Fab25 during the binding process.

**Non-competitive inhibition mechanism of Fab25**

Eadie–Hofstee plots of results from hepsin enzyme kinetics experiments with the P3–P1 substrate S2765 indicated a non-competitive inhibition mechanism, as increasing Fab25 concentration reduced $V_{app\,\max}$ values, while $K_{app}$ values remained unchanged (Fig. 5A). Similar results were obtained with the substrate Ac-KTKQLR-amc (P6–P1 of pro-HGF) (data not shown). The P2–P1 and P1 substrates Z-Lys-Arg-pNA and Bz-Arg-pNA were poor substrates, precluding detailed enzyme kinetics. However, Fab25 fully inhibited hydrolysis and velocity vs [substrate] plots were indicative of non-competitive inhibition, as there were strong effects on $V_{app\,\max}$ values and little or no changes on $K_{app}$ values (Fig. 5B and C). The $V_{app\,\max}$ values of Fab25 control, 10 nM Fab25 and 25 nM Fab25 were 11.86, 5.48 and 2.64 $\mu$M/min for Z-Lys-Arg-pNA and 3.87, 2.12 and 1.29 $\mu$M/min for Bz-Arg-pNA. The corresponding $K_{app}$ values were 3.8, 3.3 and 4.2 mM for Z-Lys-Arg-pNA and 1.2, 1.4 and 1.6 mM for Bz-Arg-pNA.

**Discussion**

Antibodies are widely used as therapeutic agents and are exquisite tools to study protein function in biological
pathways. Due to their versatility in protein interactions and ability to discriminate between conserved topologies of protease family members, antibodies are ideally suited to specifically inhibit the activity of extracellular proteases, such as hepsin. In principle, antibodies may interfere with the activity of trypsin-like serine proteases by many different mechanisms, all of which can be very effective in neutralizing proteolytic function. These mechanisms include active site-directed steric and allosteric mechanisms (Colwell et al., 1998; Wu et al., 2007; Farady et al., 2008; Ganesan et al., 2007, 2010), inhibition of zymogen activation (Blouse et al., 2009; Botkjaer et al., 2011), interference with macromolecular substrate interaction (exosite) (Dickinson et al., 1998; Botkjaer et al., 2011), inhibition of protease binding to cofactors or phospholipids (Carson et al., 1985; Refino et al., 1999; Huang et al., 2004) or cofactor interaction with substrates (Huang et al., 1998). Intriguingly, some antibodies were found to utilize more than one inhibition mechanism, as exemplified by the anti-uPA antibody mAb-112, which prevents pro-uPA activation and inhibits catalytic activity of the active form by stabilizing it in a zymogen-like state (Blouse et al., 2009).

Recent structural studies revealed the molecular details of active site-directed mechanisms by several anti-serine protease antibodies (Wu et al., 2007; Farady et al., 2008; Ganesan et al., 2009). Some protease/Fab complex X-ray structures showed that unlike standard-mechanism inhibitors, such as Kunitz domains, the inhibitory mechanism does not involve antibody interaction with the S1 pocket of the protease (Shia et al., 2005; Wu et al., 2007; Ganesan et al., 2009). Therefore, we reasoned that occupancy of the S1 pocket by a small inhibitor, such as the mechanism-based inhibitor DCI would not impede antibody binding to hepsin, while improving the chances of finding inhibitory antibodies, due to stabilizing effects on the active site. This strategy led to the identification of Fab25 by use of a constrained Fab phage display library. An added benefit of this approach is that Fab25 was expected to be safe-guarded from proteolytic processing, since the S1 interaction is critical for catalysis. Binding experiments with DCI-blocked hepsin confirmed that Fab25 binding was not mediated by S1 interaction, since if bound equally well to both ‘free’ hepsin and DCI-blocked hepsin (data not shown). In accord, we found that purified Fab25 was completely resistant to proteolysis by hepsin, even though the unusually long CDR-H3 loop contains several potential hepsin cleavage sites. The anti-MT-SP1 antibody E2 also has a very long CDR-H3, which unlike Fab25, occupies the S1 pocket and undergoes cleavage, albeit at a very slow rate (Farady et al., 2007).

The elucidated non-competitive inhibition mechanism of Fab25 indicates that the binding site is not centered at the active site of hepsin, since this would result in steric hindrance of substrate access and manifest itself as a competitive inhibition system as seen for FabE2 and Fab58 (Wu et al., 2007; Farady et al., 2008). Rather, the findings suggest that Fab25 is an allosteric inhibitor acting by a mechanism that perturbs the catalytic machinery. Enzyme kinetics showed that, in addition to the non-competitive inhibition of pNA substrates occupying S3-S1 (e.g., S2765) and S2-S1 (Z-Lys-Arg-pNA), Fab25 also non-competitively inhibited hydrolysis of Bz-Arg-pNA that only occupies the S1 subsite. Therefore, Fab25 imposes allosteric influences that are directed toward the core of the catalytic machinery, which may potentially include conformational disturbances at the S1 pocket itself, the oxyanion hole, the catalytic triad or, indirectly, the N-terminal insertion into the activation pocket. These structural elements are formed by the intrinsically mobile loops that undergo conformational rearrangements during the zymogen to enzyme transition (Huber and Bode, 1978). We observed differential binding of Fab25 to an engineered single-chain hepsin versus active hepsin. Our engineered single-chain hepsin has an endorexis (DDDDK ↓I) site in place of the native activation site (PVDR ↓I). Assuming that these changes have minimal influence on Fab25 binding, this would indicate that Fab25 recognizes one or more of these loops as part of its binding site to impose its allosteric effect. Blouse et al. (2009) described an anti-uPA antibody mAb-112 that binds to one of these mobile loops (the autolysis loop = 140-loop) to impart enzyme inhibition by a non-competitive mechanism. However, unlike anti-hepsin Ab25, the mAb-112 binds with high affinity to the zymogen form and impairs zymogen conversion to the active enzyme, indicating divergent inhibition mechanisms of the two antibodies. Moreover, thermodynamic measurements of Fab25–hepsin interaction suggested enthalpically driven binding, perhaps contributed by interactions of charged residues, many of which are clustered on CDR-H3 (Fig. 1). Clearly, only the crystal structure of the Fab25:hepsin complex will provide the molecular details of this intriguing inhibition mechanism. Unfortunately, our efforts to obtain a crystal structure were not successful so far.

The detrimental effect on catalysis by Fab25 is underscored by its complete inhibition of hepsin enzymatic activity toward four macromolecular and seven synthetic substrates. Also, Fab25 is highly specific for hepsin, since it does not affect the enzymatic activity of nine selected trypsin-like serine proteases, some of which are close homologues of hepsin. Therefore, Ab25 constitutes a valuable new tool to further investigate hepsin’s role in tumor biology, since it alleviates the shortcomings of previously described inhibitors with respect to potency and specificity, and also has potential therapeutic applications. In addition, the study shows that antibody phage display with active site modified protease as the ‘bait’ may select for allosteric inhibitors and, thus, may be applicable to proteases across different mechanistic classes.

**Supplementary data**

Supplementary data are available at PEDS online.

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