Blocking the proteolytic activity of zymogen matriptase with antibody-based inhibitors

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Matriptase is a member of the type-II transmembrane serine protease (TTSP) family and plays a crucial role in the development and maintenance of epithelial tissues. As all chymotrypsin-like serine proteases, matriptase is synthesized as a zymogen (proform), requiring a cleavage event for full activity. Recent studies suggest that the zymogen of matriptase possesses enough catalytic activity to not only facilitate autoactivation, but also carry out its in vivo functions, which include activating several proteolytic and signaling cascades. Inhibition of zymogen matriptase may therefore be a highly effective approach for limiting matriptase activity. To this end, here we sought to characterize the catalytic activity of human zymogen matriptase and to develop mAb inhibitors against this enzyme form. Using a mutated variant of matriptase in which the serine protease domain is locked in the zymogen conformation, we confirmed that the zymogen form of human matriptase has catalytic activity. Moreover, the crystal structure of the catalytic domain of zymogen matriptase was solved to 2.5 Å resolution to characterize specific antibody-based matriptase inhibitors and to further structure-based studies. Finally, we describe the first antibody-based competitive inhibitors that target both the zymogen and activated forms of matriptase. We propose that these antibodies provide a more efficient way to regulate matriptase activity by targeting the protease both before and after its activation and may be of value for both research and preclinical applications.

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This article contains Table S1 and Figs. S1–S4.

This work is dedicated to our dear colleague and friend, Prof. Peter A. Andrea-...
which a lysine side chain near the activation hole in the catalytic domain is able to substitute for the hydrogen bond that is normally not formed until activation cleavage results in the insertion of a newly released N terminus (19). Interestingly, the zymogen form of rat matriptase has been shown to harbor significant catalytic activity, although no obvious structural or sequence-based reason has yet been proposed (20). Several studies have also provided strong evidence for human zymogen matriptase being able to autoactivate, suggesting a basal activity for the matriptase zymogen (21, 22). Furthermore, we have previously shown that the zymogen form of matriptase reacts with a peptide inhibitor biotin-Arg-Gln-Arg-Arg chloromethyl ketone (CMK) (23). Finally, it was recently shown that a nonactivatable (zymogen-locked) version of mouse matriptase was able to support the natural function of matriptase in a transgenic mouse model system where natural matriptase had been ablated, in strong support of a physiological role of zymogen activity of matriptase (24).

As matriptase is an attractive molecular target in basic research and pharmacological intervention, there has been much interest in developing inhibitors. Currently, several active-site inhibitors against matriptase have been engineered, with varying success due to lack of affinity, specificity, or both. Active-site inhibitors include modified naturally occurring cyclopeptides and several monoclonal antibodies (25–27). To date, only one set of compounds has been reported to target the zymogen form of matriptase, the small organochemicals termed compounds 1–4, which all showed an improved inhibitory effect toward matriptase-mediated activation of prostasin as compared with an active-site inhibitor of activated matriptase. However, the reported IC50 values were in the low micromolar range, the best being 2.6 μM, and the information regarding specificity and mechanism remains to be addressed (28).

The present study was initiated to investigate the catalytic activity of the zymogen form of human matriptase. We show that a zymogen-locked mutant of human matriptase has significant catalytic activity, although no obvious structural or sequence-based reason has yet been proposed (20). Several studies have also provided strong evidence for human zymogen matriptase being able to autoactivate, suggesting a basal activity for the matriptase zymogen (21, 22). Furthermore, we have previously shown that the zymogen form of matriptase reacts with a peptide inhibitor biotin-Arg-Gln-Arg-Arg chloromethyl ketone (CMK) (23). Finally, it was recently shown that a nonactivatable (zymogen-locked) version of mouse matriptase was able to support the natural function of matriptase in a transgenic mouse model system where natural matriptase had been ablated, in strong support of a physiological role of zymogen activity of matriptase (24).

Results

The zymogen form of matriptase displays catalytic activity

To characterize the catalytic activity of matriptase in its zymogen form, we prepared a zymogen variant of the serine protease domain (SPD) by recombinant expression in yeast (Pichia pastoris). The sequence Cys604–Val855 of human matriptase was fused to an N-terminal His tag, and the muta-
tion N164Q was introduced to avoid N-glycosylation. An additional mutation in the activation cleavage site (R614A) created a variant of matriptase locked in the zymogen form (Fig. 1B). The resulting 29-kDa recombinant protein was used as a representative of the zymogen form of matriptase termed zSPD. Zymogen purity is >95% as demonstrated by a Coomassie-stained SDS-polyacrylamide gel (Fig. 1C). The WT SPD of matriptase was expressed in Escherichia coli and refolded in vitro. As this recombinant matriptase spontaneously autoactivated during purification, as was observed by SDS-PAGE band shift and verified by MS and chromogenic activity assays (data not shown), this is henceforth used as a representative of the activated form of matriptase termed acSPD. Hydrolysis of the chromogenic peptide substrate, isoleucil-prolyl-arginine-p-nitroaniline (S-2288, Chromogenix) by either zSPD or acSPD was measured, and the corresponding enzymatic parameters $K_m$ and $k_{cat}$ were calculated (Table 1 and Fig. 2A). The catalytic efficiencies ($k_{cat}/K_m$) were determined to be $3.0 \times 10^7 \pm 7 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and $1.01 \times 10^7 \pm 217 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ for zSPD and acSPD, respectively. By calculating the ratio of the catalytic efficiencies, a zymogenicity factor of 33 ± 11 was obtained, which corresponds to zSPD having an apparent catalytic activity of ~3% of that of acSPD.

**Characterization of mAb-based inhibitors of zymogen and activated matriptase**

Monoclonal antibodies were generated with zSPD as antigen. A panel of nine monoclonal antibodies (aZ-mAb-1 to -7, aZ-mAb-9, and aZ-mAb-10) was chosen for further examination. Surface plasmon resonance (SPR) was used to analyze the binding of the antibodies to zymogen and activated matriptase. All nine monoclonal antibodies bound both zSPD and acSPD with affinities ($K_{\text{d}}$) in the low nanomolar range (Table S1, Fig. 3, and Figs. S1 and S2). The nine monoclonal antibodies underwent further testing to establish any modulatory effect on the catalytic activity of zymogen or activated matriptase. The catalytic activity of zSPD or acSPD toward a peptide substrate was measured in the presence of each antibody (Fig. 2B). Our initial results showed that two antibodies, aZ-mAb-6 and aZ-mAb-7, dose-dependently inhibited the activity of both zSPD and acSPD. The remaining antibodies (aZ-mAb-1, -2, -3, -4, -5, -9, and -10) caused either no inhibition or only partial inhibition.

To characterize the mode of inhibition of the antibodies, the $V_{max}$ and $K_m$ of acSPD in the presence or absence of antibodies were determined (Table 2 and Figs. S3 and S4). We observed an unchanged $V_{max}$ and an increased $K_m$ in the presence of aZ-mAb-6 and aZ-mAb-7, suggesting a competitive mechanism of inhibition (24). For the antibodies aZ-mAb-1, -2, -3, -5, -9, and -10, little change in $K_m$ and $V_{max}$ was detected (Table 2), suggesting that they are noncompetitive or mixed inhibitors. As no change was observed for $K_m$ and $V_{max}$ in the presence of antibody aZ-mAb-4, the lack of inhibitory activity of this antibody was confirmed, and it was used as a comparative control in subsequent experiments. Next, the equilibrium constant ($K_i$) of aZ-mAb-6 and aZ-mAb-7 against activated matriptase was determined to 21 ± 2 and 9 ± 2 nM, respectively (Fig. 2C). A similar analysis was performed on zSPD, including aZ-mAb-6 and aZ-mAb-7 (Table 2). Again, we observed an unchanged $V_{max}$ and an increased $K_m$ in support of a competitive mechanism of inhibition for aZ-mAb-6 and aZ-mAb-7. As expected, there was no significant effect of aZ-mAb-4.

**Antibodies aZ-mAb-6 and aZ-mAb-7 are not substrates for matriptase**

To ensure that the difference in affinity of the antibodies toward acSPD and zSPD is not due to antibody cleavage catalyzed by matriptase, the stability of the antibodies was analyzed following prolonged incubation with matriptase. Antibodies aZ-mAb-6 and aZ-mAb-7 were incubated with acSPD at a 1:1 molar ratio before SDS-PAGE analysis. The gel showed no sign of matriptase-catalyzed antibody degradation (Fig. 4).

**Antibodies aZ-mAb-6 and aZ-mAb-7 target human matriptase with high specificity**

The active sites of serine proteases are generally highly conserved, and therefore inhibitory compounds often inhibit several related proteases. To gain insight about the inhibitory specificity of antibodies aZ-mAb-6 and aZ-mAb-7, three human serine proteases closely related to matriptase were tested: urokinase plasminogen activator (uPA), hepatocyte growth factor activator, and hepsin. Even at an antibody concentration of 400 nM, a 20-fold higher concentration than the inhibitory constant ($K_i$) toward matriptase, no inhibition was observed (Fig. 5). Furthermore, no inhibition was observed toward the mouse ortholog of matriptase (Fig. 5), whose catalytic domain shares 87% identity to the human protein. Together, these observations strongly suggest that antibodies aZ-mAb-6 and aZ-mAb-7 have high specificity toward human matriptase.

**Antibodies aZ-mAb-6 and aZ-mAb-7 can identify and block the activity of WT full-length matriptase in complex biological samples**

To examine the potential use of antibodies aZ-mAb-6 and aZ-mAb-7 for the study of matriptase activity in complex biological samples, an assay was established using cultured cells. HEK293 cells were transiently transfected with combinations of WT full-length membrane-anchored matriptase and two variants of its cognate inhibitor HAI-2. Matriptase needs co-expression with the inhibitors HAI-1 or HAI-2 to obtain significant levels of plasma membrane-bound matriptase. Either WT HAI-2 or a HAI-2 variant with compromised inhibitory activity (HAI-2 C47F) due to a mutation in the predicted P2 position of the inhibitory loop of Kunitz domain 1 was used (29). Transiently transfected cells were lysed, and the peptidolytic activity of the cell lysates was assayed with or without the antibodies aZ-mAb-6 and aZ-mAb-7 or a control antibody (anti-uPA).
Both antibodies aZ-mAb-6 and aZ-mAb-7 but not the control antibody inhibited the proteolytic turnover of the broad-spectrum serine protease substrate; this activity was interpreted as derived from unopposed matriptase in either the active zymogen form or the activated form. Little or no background activity was observed in mock-transfected cells or cells transfected with matriptase and WT HAI-2, suggesting that little to no unopposed matriptase is present under these conditions.

Antibodies aZ-mAb-6 and aZ-mAb-7 can inhibit matriptase catalyzed cleavage of a natural protein substrate

To examine the ability of antibodies aZ-mAb-6 and aZ-mAb-7 to inhibit the cleavage of a natural matriptase substrate, a pro-HGF cleavage assay was established. Pro-HGF was incubated with or without zSPD or acZPD in the presence of varying concentrations of antibodies. The results showed that aZ-mAb-6 and aZ-mAb-7 were the most efficient inhibitors. The values for apparent $K_i$ were determined by fitting the calculated reaction rates to Equation 2 (right) for competitive inhibition and then converting into the $K_i$ value using Equation 3.

Figure 2. The activity of acSPD and zSPD and the corresponding inhibitory effects of monoclonal antibodies on activity. A, activity of acSPD and zSPD shown by a dose-dependent enzyme activity assay (top left and bottom left, respectively) and the corresponding Michaelis–Menten fit (right panels) for 1 nM acSPD (top right) and 10 nM zSPD (bottom right) using 0–0.4 or 2 mM S-2288 substrate. Each graph is one representative experiment from at least three independent experiments. The zSPD has a lower $K_m$ and $k_{cat}$ than acSPD as a consequence of the higher concentration and lower activity. B, initial screening of inhibitory effects of the nine monoclonal antibodies toward both acSPD (left) and zSPD (right). An increasing concentration of antibody was incubated with either acSPD (50 pM) or zSPD (20 nM), and the residual rate of substrate conversion was measured by chromogenic assays ($n = 3$ for each data point of the shown curves). Antibodies aZ-mAb-6 and -7 are the most efficient inhibitors. C, $K_i$ determinations of aZ-mAb-6 and aZ-mAb-7. acSPD (50 pM) was preincubated with a concentration series of the antibodies for 1 h, and the conversion of the chromogenic substrate S-2288 was monitored as the change in absorbance at 405 nm at 37 °C for 1 h (left). The $K_i$ values for aZ-mAb-6 and -7 were 21 ± 2 and 9 ± 2 nM, respectively. The values determined by fitting the calculated reaction rates to Equation 2 (right) for competitive inhibition and then converting into the $K_i$ value using Equation 3.
Investigated whether mutation of the nucleophilic serine 805 were only carried out only on activated protein. First, it was matriptase, which is why some of the following experiments aZ-mAb-7 are conserved between zymogen and activated protein substrate.

We presume that the epitopes of antibodies aZ-mAb-6 and aZ-mAb-7 are near the active site to directly compete with substrates.

Inhibition of zymogen matriptase activity

Antibodies aZ-mAb-6 and aZ-mAb-7 bind near the active site of matriptase.

Or absence of inhibitors. Both antibodies aZ-mAb-6 and aZ-mAb-7, but not the control antibody (anti-uPA), inhibited the cleavage of pro-HGF by both acSPD and zSPD into its α and β chains (Fig. 6B, top and bottom, respectively). A peptide inhibitor, biotin-RQRR-CMK (23), was included as a positive control. These results strongly suggest that antibodies aZ-mAb-6 and aZ-mAb-7 inhibit matriptase-catalyzed cleavage of a physiologically relevant protein substrate.

**Antibodies aZ-mAb-6 and aZ-mAb-7 bind near the active site of matriptase**

As competitive inhibitors, we expect both antibodies to bind in or near the active site to directly compete with substrates. We presume that the epitopes of antibodies aZ-mAb-6 and aZ-mAb-7 are conserved between zymogen and activated matriptase, which is why some of the following experiments were only carried out only on activated protein. First, it was investigated whether mutation of the nucleophilic serine 805 (Ser195) in the active site of matriptase affected binding of the antibodies to zymogen or activated matriptase. Using SPR, no differences were seen in the $K_D$ for binding of antibodies aZ-mAb-6 and aZ-mAb-7 to zSPD, zSPD-S805A, or their activated counterparts (data not shown). This suggests that antibodies aZ-mAb-6 and aZ-mAb-7 do not interact directly with the nucleophile serine.

Next, possible interaction between the antibodies and the specificity pockets S2–S4 of matriptase, as S1 is not present in the zymogen, was probed by competition with either the peptide inhibitor biotin-RQRR-CMK (23) or the isolated inhibitor domain of HAI-1 termed IK1 (30). The peptide CMK inhibitor is a small molecule inhibitor of matriptase that binds covalently to the reactive histidine residue in the active site of matriptase via its CMK, whereas its peptide moiety is expected to occupy the S1–S4 specificity pockets adjacent to the active site. Neither aZ-mAb-6 nor aZ-mAb-7 bound to acSPD in the presence of

| Table 2 |
| Summary of $V_{max}$, $K_m$, $k_{cat}$, and $K_i$ |

|        | $V_{max}$ | $K_m$ | $k_{cat}$ | $K_i$ | $n$ |
|--------|-----------|-------|-----------|-------|-----|
| acSPD  |           |       |           |       |     |
| No antibody | 391 ± 38  | 387 ± 74 | 39 ± 4    | NA$^a$ | 5   |
| aZ-mAb-1 | 184 ± 38$^b$ | 197 ± 64 | 18 ± 4    | NA     | 3   |
| aZ-mAb-2 | 348 ± 22  | 489 ± 45 | 35 ± 2    | NA     | 3   |
| aZ-mAb-3 | 152 ± 27$^b$ | 166 ± 36 | 15 ± 3    | NA     | 3   |
| aZ-mAb-4 | 349 ± 61  | 347 ± 87 | 35 ± 6    | NA     | 4   |
| aZ-mAb-5 | 339 ± 60  | 421 ± 102 | 34 ± 6   | NA     | 3   |
| aZ-mAb-6 | 371 ± 35  | 857 ± 84$^b$ | 37 ± 4 | 21 ± 2 | 3   |
| aZ-mAb-7 | 365 ± 19  | 1172 ± 255$^b$ | 37 ± 2 | 9 ± 2 | 3   |
| aZ-mAb-9 | 281 ± 58$^a$ | 424 ± 109 | 28 ± 6 | NA | 3 |
| aZ-mAb-10 | 308 ± 42$^b$ | 451 ± 102 | 31 ± 4 | NA | 3 |

$^a$ NA, not applicable. 
$^b$ Statistically significant difference from “no antibody” by unpaired t test calculated using GraphPad QuickCalcs online software.
the CMK inhibitor (Fig. 7A), whereas binding of the control noninhibitory antibody aZ-mAb-4 was not affected. The IK1 domain of HA1-1 binds with high affinity to the active site and covers an extended area in and around the active site and specificity pockets of matriptase (30, 31). SPR analysis showed that IK1 blocked the binding of not only aZ-mAb-6 and aZ-mAb-7 but also aZ-mAb-4 to acSPD (Fig. 7B). This suggests that all three epitopes must be near the active site of matriptase, as the larger footprint of IK1 prevents binding of all three antibodies. Finally, the three antibodies were shown to have overlapping epitopes by SPR, as each of the three antibodies block mutual binding of the other antibodies to zSPD (Fig. 7C).

**The structure of zymogen matriptase reveals a classical chymotrypsin-like zymogen fold**

With the availability of zymogen-locked matriptase protein, the X-ray crystal structure was pursued with the intent to provide structural insight into the unusual activity of the zymogen as well as aid in the characterization of our antibodies. To prevent unwanted proteolytic degradation during crystallization, an additional mutation of the nucleophile Ser805 (Ser195) to alanine was introduced (zSPD-S805A, Fig. 1D). The atomic coordinates for the crystal structure are available in the Protein Data Bank under accession number 5LYO. The resulting 2.5 Å structure (Table 3) was that of a classical zymogen fold, with a strong preference for the inactivated state, whereas the control noninhibitory antibody aZ-mAb-4 was not affected. The IK1 domain of HA1-1 binds with high affinity to the active site and covers an extended area in and around the active site and specificity pockets of matriptase (30, 31). SPR analysis showed that IK1 blocked the binding of not only aZ-mAb-6 and aZ-mAb-7 but also aZ-mAb-4 to acSPD (Fig. 7B). This suggests that all three epitopes must be near the active site of matriptase, as the larger footprint of IK1 prevents binding of all three antibodies. Finally, the three antibodies were shown to have overlapping epitopes by SPR, as each of the three antibodies block mutual binding of the other antibodies to zSPD (Fig. 7C).

**Inhibition of zymogen matriptase activity**

**Figure 5. The monoclonal antibodies aZ-mAb-6 and -7 specifically inhibit human matriptase.** The ability of aZ-mAb-4, -6, and -7 to inhibit human matriptase and other closely related serine proteases was tested in an activity assay. For all assays, an excess of antibody (400 nM) was preincubated with the proteases for 1 h at 37 °C before the addition of substrate. Human matriptase, acSPD (100 pm), was almost completely inhibited by aZ-mAb-6 (green line) and aZ-mAb-7 (black line), but not by aZ-mAb-4 (red line). The activity of mouse matriptase SPD (100 pm), human uPA (1 nm), hepsin (1 nm), and HGFA (5 nm) were not significantly inhibited by any of the monoclonal antibodies. The graphs shown are representative of three replicates.

The match between the activation motif and the substrate specificity of matriptase itself, a unique feature among serine proteases, may be transactivating other matriptase zymogens due to the matching between the activation motif and the substrate specificity of matriptase itself, a unique feature among serine proteases (21, 33). This is also in line with the matriptase SPD being fully activated during the in vitro refolding procedure, as observed in our previous study (30).

According to the paradigm, a zymogen is thought to be in constant conformational transition equilibrium between two structural states with a presumed short-lived “active-like” conformation (34). Chymotrypsin-like serine proteases show strong preference for the inactive state and are thus virtually inactive (e.g. trypsinogen). For a rare subset of these proteases,
Inhibition of zymogen matriptase activity

Figure 6. A, the monoclonal antibodies aZ-mAb-6 and -7 inhibit matriptase activity in the protein extracts of transiently transfected cells. HEK293 cells were transiently transfected with empty expression vector (mock) or expression vectors encoding WT full-length matriptase together with WT HAI-2 or mutant HAI-2 C47F, respectively. Protein extracts were preincubated for 1 h at room temperature with 500 ng of antibody (anti-uPA, aZ-mAb-6, aZ-mAb-7, or no antibody), and a peptidolytic activity assay was subsequently carried out with 300 μM chromogenic substrate S-2288. The absorbance of the reaction mixture was measured at 405 nm continuously every 5 min for 5 h, and the mean velocity of the substrate reaction (in milli-absorbance units/min) was calculated. The figure shown is representative of three replicates. B, aZ-mAb-6 and -7 inhibit matriptase-mediated pro-HGF cleavage. Pro-HGF (60 nM final concentration) was incubated for 4.5 h at 37 °C with either 200 nM zSPD or 1 nM acSPD that had been preincubated with aZ-mAb-6, aZ-mAb-7, anti-uPA, or biotin-RQRR-CMK, as indicated above the gels. The processing of pro-HGF was visualized on Western blotting using an antibody that recognizes pro-HGF as well as its α and β chains. Antibody-only samples were prepared in parallel to show the cross-reactivity of the antibodies themselves with the secondary antibody.

The equilibrium appears to be shifted toward a more active-like zymogen population.

It is clear that many cells have zymogen matriptase on the plasma membrane and that this may have enough catalytic activity to control the activation of growth factors, signaling pathways, etc. to sustain survival (24, 32). Our working hypothesis is that zymogen matriptase, which is found in low concentrations on the cell surface, is a pinnacle protease in multiple cascades with enough catalytic activity to perform its physiological role. If the concentration of zymogen matriptase increases on the cell surface, they will begin to encounter one another, resulting in possible transactivation events. The activated matriptase molecules will then be immediately inhibited by its co-expressed (co-localized) inhibitors HAI-1/HAI-2 and targeted for endocytosis and degradation. Solid evidence suggests that if the equilibrium between matriptase and HAI-1/HAI-2 is out of balance in such a way that the total matriptase activity (sum of activity of zymogen and activated matriptase) becomes dominating, this accumulation of unopposed matriptase activity results in carcinogenesis (3, 35). Studies in mice have shown that both HAI-1 and HAI-2 can negate the oncogenic potential of matriptase when overexpressed together (13, 14).

In this study, we have generated two competitive antibodies (aZ-mAb-6 and aZ-mAb-7) that target the activity of both zymogen and activated matriptase. These inhibitory molecules represent, to the best of our knowledge, the first macromolecular inhibitors of any chymotrypsin-like serine protease in the zymogen form. The antibodies are specific for human matriptase, bind with affinities in the low nanomolar range, and are capable of inhibiting both glycosylated (full-length matriptase from a HEK293 cell culture system; Fig. 6A) and nonglycosylated matriptase. Although our antibody-based inhibitors may exhibit lower affinity relative to a naturally occurring Kunitz-type inhibitor such as HAI-1 or will eventually lose in competition with a covalent CMK-based inhibitor, the unmatched specificity of an antibody outweighs all when studying or targeting the activity of matriptase activity in complex mixtures such as in vivo studies or in a clinical setting. It is likely that our antibodies can be used to study the in vivo importance of the activation process, as we expect these antibodies to block transactivation on the cell surface. Furthermore, aZ-mAb-6 and aZ-mAb-7 could possibly be used in research and diagnostics to evaluate the level of unopposed matriptase activity (matriptase activity not inhibited by inhibitors) in complex biological samples, as shown in Fig. 6, which has not been possible previously due to the lack of a specific substrate and/or inhibitors. The inhibitory antibodies described in the present study will also prove a tool to elucidate whether inhibition of plasma membrane-bound zymogen matriptase or activated matriptase is sufficient to prevent/inhibit cancer development.

The solved crystal structure of zymogen matriptase has an overall conformation that resembles the classical chymotrypsin-like zymogen form with loops in and around the active site deviating in conformation from that of the active structure, a zymogen triad and no oxyanion hole or S1 specificity pocket. In all, the structure of zymogen matriptase suggest that the crystallized form represents the inactive form of the unactivated zymogen and that future studies must be done to describe the apparent conformational liability of the zymogen form of matriptase that allow for its unusually high zymogen activity.

The structural information from available structures of the catalytic domain of active and now zymogen matriptase was combined with the observations from the biochemical and biophysical analyses to propose the possible epitopes of the antibodies. Comparable binding affinities of the antibodies against both forms of matriptase suggest that the antibodies mainly...
bind in a region of the structure with a conformation that is invariant before and after activation. Comparing the zymogen and active structure (Fig. 8D), the N-terminal subdomain appears to present the most promising invariant area near the active site. The antibodies do not appear to bind directly in the active site because binding is unaffected by mutation of the active-site serine. However, reduced binding to acSPD in the presence of the biotin-RQRR-CMK inhibitor showed that aZ-mAb-6 and aZ-mAb-7 most likely bind in close proximity to the active site. The fact that the antibodies bind zSPD to the same response on the surface plasmon resonance sensor chip (total associated mass) as acSPD and that both zymogen and activated matriptase are inhibited suggests that the antibodies must bind both the “active-like” zymogen conformation and the prevailing inactive zymogen conformation. This means that inactive zymogen can interact with the inhibitory antibodies even before transition to the “active” zymogen form has taken place, which makes them very efficient inhibitors. This also suggests that binding of the antibodies does not require the presence of the S1 pocket, which is not formed in the true zymogen form, thus excluding the area around the S1 pocket from the epitopes. This is a rather unusual feature for a competitive inhibitor of a serine protease, which often relies on direct interaction with the S1 pocket for specificity and affinity (36). Furthermore, based on the fact that the epitope of aZ-mAb-4 overlaps the epitopes of both aZ-mAb-6 and aZ-mAb-7 and all three antibodies compete with binding of the IK1 fragment of HAI-1, we propose that aZ-mAb-6 and aZ-mAb-7 are binding mainly to the N-terminal conformationally invariant subdomain near, but not in, the S1 pocket, effectively covering most of the noncatalytic parts of the active site of matriptase. This is also compatible with our observation that both antibodies inhibit matriptase glycosylated on the α-helix adjacent to the 170 loop (Fig. 8D, top right). During this study, an extensive attempt to map the exact

Figure 7. Epitope mapping of the monoclonal antibodies by SPR. A and B, antibodies (labeled above each panel) were captured by anti-mouse IgG directly immobilized on a CM5 chip before injection of the active site–binding peptide biotin-RQRR-CMK (A) or the larger active site–binding protein IK1 (B) in complex with acSPD. Both biotin-RQRR-CMK and IK1 block the binding of acSPD to aZ-mAb-6 and aZ-mAb-7. IK1 also blocks binding of acSPD to aZ-mAb-4. C, the antibodies aZ-mAb-4, -6, and -7 (labeled above) were individually immobilized on a CM5 chip surface, and antibody alone or in complex with zSPD was injected over the surface-bound antibodies. Antibodies injected alone as a control did not show any observable binding (not shown). Antibody was incubated with zSPD for 1 h at room temperature. All zSPD–antibody complexes have reduced binding, indicating that aZ-mAb-4, -6, and -7 have overlapping epitopes. The shown sensorgrams are representative of at least three replicates.

Table 3
Data collection and refinement statistics
Values in parentheses are for the highest-resolution shell.

| Data collection          |       |
|-------------------------|-------|
| PDB accession code      | 5LYO  |
| Wavelength (Å)          | 0.97625 |
| Space group             | P3121 |
| a, b, c (Å)             | 108.50, 108.50, 123.70 |
| α, β, γ (degrees)       | 90, 90, 120 |
| Resolution (Å)          | 50–2.50 (2.65–2.50) |
| Rmerge                  | 0.194 (1.116) |
| fcalc                   | 6.08 (1.27) |
| Completeness (%)        | 97.6 (89.5) |
| Redundancy              | 2.88 (2.58) |
| No. of unique reflections| 56,209 (8340) |
| CC(1/2)                 | 0.998 (0.529) |

| Refinement              |       |
|-------------------------|-------|
| R factor                | 0.2144 |
| Rfree                   | 0.2629 |
| Root mean square deviation | 0.020 |
| Bond lengths (Å)        | 1.998 |
| Ramachandran plot (%)   |       |
| Favored                 | 95.89 |
| Allowed                 | 5.31  |
| Outliers                | 0.80  |

Inhibition of zymogen matriptase activity

J. Biol. Chem. (2019) 294(1) 314–326 321
Inhibition of zymogen matriptase activity

Figure 8. The structure of zymogen matriptase, zSPD-S805A. A, the solved structure of zSPD-S805A (wheat) with the 60 loop, the 70 loop, the 170 loop, and the activation loop specified together with the residues in the catalytic triad and the S1 pocket (PDB entry 5LYO). B, the intact activation loop for zSPD-S805A (wheat) and the cleaved activation loop for activated matriptase (red). C, the zymogen triad for zSPD-S805A and activated matriptase, showing rearrangement of Asp604 (Asp194). D, alignment of zSPD-S805A (wheat) and activated matriptase (gray), where the loops undergoing conformational changes are marked in red for activated matriptase. The S1 pocket is not formed in the zymogen.

epitopes was carried out; however, mutating solvent-exposed side chains in the area surrounding the active site of matriptase resulted in loss of either protein expression or folding efficiency (data not shown). Future co-crystallization studies may be able to reveal more structure-based insight into epitopes and the inhibitory mechanism of the antibodies.

Experimental procedures

Recombinant proteins

Production of activated matriptase catalytic domain (acSPD) in E. coli—A pT7 vector containing the human matriptase gene, GenBank™ accession NG_012132.1 and a C602S mutation to remove the cysteine between the activation loop and SPD was transformed into competent BL21 (DE3) E. coli cells (30). Cells were cultured under agitation at 37°C in 2xYT media supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. A 20-ml overnight culture was transferred into 1 liter of 2xYT medium and grown until the absorbance at 600 nm was >0.6 before induction with 1 mM isopropyl-D-1-thiogalactopyranoside for 4 h. The cells were pelleted by centrifugation at 5000 × g for 20 min and frozen at −80°C until purification.

Purification and refolding of the catalytic domain of matriptase (acSPD) from E. coli—Cell pellets were resuspended in sonication buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA) and sonicated on ice. Lysates were pelleted at 10,000 × g for 30 min, and the insoluble fraction was washed in several steps using wash buffer 1 (50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1% Triton X-100), wash buffer 2 (50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.25% Triton X-100), and wash buffer 3 (50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA). After each wash, the sample was centrifuged as described above, and the supernatant was discarded. The insoluble matter was resuspended in denaturing buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol containing 6 M urea, 1 mM EDTA) by slowly stirring on ice for 30 min. The resuspended pellet was then centrifuged as described above, and the supernatant was incubated with nickel-nitritotriacetic acid–agarose containing 20 mM imidazole by slow stirring on ice for 1 h. Elution was performed with denaturing buffer supplemented with 400 mM imidazole, and fractions containing protein were dialyzed against denaturing buffer. The protein concentration was measured at an absorbance of 280 nm with an extinction coefficient of the catalytic domain of 53,200 M⁻¹ cm⁻¹. The concentrations was adjusted to 0.1–0.2 mg/ml using denaturing buffer. Protein-containing fractions were dialyzed overnight at 4°C against refolding buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM β-mercaptoethanol, and 3 M urea). Autoactivation of the protease occurs during the overnight dialysis against storage buffer (50 mM Tris, pH 8.0, 10% glycerol) at 4°C, which was confirmed by increasing activity against a chromogenic substrate and by SDS-PAGE. Activated proteins were purified over a benzamidine-Sepharose (GE Healthcare) column that had been equilibrated with storage buffer. Bound protease was eluted with storage buffer supplemented with 1 M arginine. Proteins were further purified by size-exclusion chromatography (Superdex75 HR10/30 column, GE Healthcare) using storage buffer. Protein-containing fractions were pooled, quantitated, and stored at −80°C.

Production of the catalytic domain of WT and mutant forms of zymogen matriptase in P. pastoris—The catalytic domain of WT and mutant forms of zymogen matriptase were expressed in P. pastoris. The DNA sequence encoding the matriptase catalytic domain (Cys604–Val655) was amplified from full-length human matriptase cDNA. The two mutations R614A (R15A) and N772Q (N164Q), with the corresponding standard chymotrypsin numbering given in parentheses, were introduced to lock the zymogen conformation and to remove a potential glycosylation site, respectively (zSPD). A construct with an addi-
Inhibition of zymogen matriptase activity

All SPR experiments were performed on a Biacore T200 (GE Healthcare) at 25 °C with a flow rate of 30 μl/min unless otherwise stated. A standard CM5 chip (GE Healthcare) was prepared by immobilizing covalently ~6000 response units (RU) of anti-mouse IgG antibodies (GE Healthcare) on both the active and reference flow cell according to the manufacturer's protocol (GE Healthcare). The amine coupling protocol (Biacore, GE Healthcare) was used. The individual monoclonal antibodies were captured on the active flow cell anti-mouse IgG surface in running buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.1% BSA) to ~250 RU. To correct for bulk effects, the signal from the reference surface was subtracted from the data of the active flow cell. acSPD, zSPD, and mutants thereof, were injected in varying concentrations with an association phase of 120 s and allowed to dissociate for 180 s. The anti-mouse IgG surface was regenerated between binding cycles by a 120-s pulse of regeneration buffer (10 mM glycine-HCl, pH 1.7; GE Healthcare).

For the $K_D$ determinations by SPR, six different concentrations of SPD in a 2-fold dilution series were used. The following concentrations of acSPD were used: 1.56–50 nM for aZ-mAb-6 and -7 and 0.78–25 nM aZ-mAb-4. Concentrations of zSPD were 0.94–30 nM for aZ-mAb-6 and -7.

To determine binding in the presence of the biotin-RQRR-CMK inhibitor, acSPD at a concentration of either 50 nM (for aZ-mAb-6 and -7) or 6 nM (for aZ-mAb-4) was mixed with a high excess of biotin-RQRR-CMK (5 μM) immediately before injection, as the biotin-RQRR-CMK has a short half-life in aqueous solutions. The SPD–RQRR-CMK complex or SPD–DMSO was injected over an aZ-mAb-4, -6, or -7 surface. RQRR-CMK was stored in DMSO and was therefore added in the reference SPD solution. Buffer was applied as control.

To determine binding in the presence of the IK1 inhibitor fragment of HAI-1, acSPD (50 nM) was preincubated with IK1 (100 nM) at 37 °C for 2 h. Binding of the complex was measured on either aZ-mAb-4, -6, or -7 surface. The binding was determined relative to acSPD (50 nM) and the two controls IK1 (100 nM) and HBS buffer supplemented with 1 mM CaCl₂ and 0.1% BSA.

To test for overlapping epitopes of anti-zymogen monoclonal antibodies, antibodies aZ-mAb-4, -6, and -7 and the control anti-HAI1–31 (zymate-independent antibody targeting HAI-1) were immobilized on a CM5 chip at pH 4.5 following the amine coupling protocol (GE Healthcare). The instrument was set to aim for a capture level of 1000 RU per flow cell of antibody (30 μg/ml). acSPD and zSPD (25 nM) were preincubated with the aZ-mAb-4, -6, and -7 (100 nM) for 1 h at room temperature. The matriptase–antibody complexes were added to the four antibody-immobilized flow cells and were compared against the binding of acSPD (25 nM) or zSPD (25 nM). The controls were single-antibody solutions (100 nM) and buffer. The dissociation time was increased to 600 s to ensure a clean surface.

### Purification of monoclonal antibodies aZ-mAb-4, -6, and -7

Antibodies were purified by Protein G affinity chromatography on a column equilibrated with 20 mM Tris-HCl, pH 8, 500 mM NaCl. Bound antibody was eluted with 0.1 M glycine, pH 2.5, and fractions were pH-neutralized by adding 10% of 1 M Tris-HCl, pH 9. The fractions with highest $A_{280\, nm}$ were pooled and dialyzed against 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM CaCl₂ overnight at 4 °C. Purity of the antibodies was validated by SDS-PAGE, and the antibodies were stored at 4 °C in dialysis buffer supplemented with 300 μM NaN₃.
**Inhibition of zymogen matriptase activity**

**Determination of mAb inhibition**

Unless stated otherwise, all chromogenic assays were performed at 37 °C in HBS-PEG buffer supplemented with CaCl$_2$ and PEG-8000 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 0.05% PEG 8000). Plates were read at 405 nm in a Multiskan™ GO microplate spectrophotometer (Thermo Scientific).

IC$_{50}$ determinations were performed by preincubating acSPD (50 μM) or zSPD (20 μM) with aZ-mAb-4 (0–500 nM) and aZ-mAb-6 and -7 (0–1000 nM) for 1 h at 37 °C. The reaction was initiated by adding S-2288 (500 μM) and measured for 1 h (acSPD) or 5 h (zSPD).

**Determination of the $K_m$, $V_{max}$, and $K_i$ for aZ-mAb**

The maximal velocity rate $V_{max}$ and the Michaelis constant $K_m$ are given by the equations,

$$V_0 = V_{max} \frac{[S]}{[S] + K_m} \quad \text{(Eq. 1)}$$

$$V_0 = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_{i,app}}\right)} \quad \text{(Eq. 2)}$$

$$K_i = \frac{K_{i,app}}{1 + \frac{[S]}{K_m}} \quad \text{(Eq. 3)}$$

where $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, $K_i$ is the inhibitor’s dissociation constant, $K_{i,app}$, is the apparent $K_i$, $V_0$ is the initial reaction rate, $V_{max}$ is the maximum reaction rate, and $K_m$ is the concentration of substrate needed to reach half-maximal velocity (39). All $K_m$ and $V_{max}$ determinations were made by fitting to Equation 1 in GraphPad. aZ-mAb-4 (20 nM) or aZ-mAb-6 and -7 (60 nM) were preincubated with zSPD (10 nM) or acSPD (1 nM) for 1 h before the reactions were initiated by adding S-2288 (0–2 mM). The progress of substrate cleavage was measured for 1 or 5 h for acSPD and zSPD, respectively.

**Determination of aZ-mAb inhibition constants**

For a competitive inhibitor, $K_{i,app}$ is given by Equation 2, and to correct for competition with the substrate, Equation 3 was applied to obtain the actual $K_i$ value. To determine the $K_i$ values, aZ-mAb-4, -6, and -7 (0–400 nM) were preincubated with matriptase (100 pm) for 1 h at 37 °C before the reactions were initiated with S-2288 (500 μM). The reaction progress was monitored for 1 h.

**Determination of specificity of aZ-mAbs**

Specificity assays were carried out in HBS-PEG buffer or HBS-BSA buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 0.1% BSA). The proteases and their respective chromogenic substrates in HBS-PEG buffer were as follows: acSPD (100 μM) and mouse matriptase catalytic domain (100 μM; R&D Systems) with S-2288 (500 μM); full-length human uPA (1 nM; ProSpec) with S-2444 (500 μM); full-length human HGFA (5 nM; Sino Biological Inc.) with Spectrozyme FVIIa (500 μM), where HGFA was activated by thrombin (1:100 molar ratio) at 37 °C for 1 h until thrombin was blocked with 10 μg/ml Dextran Sulfate; and human hepsin (1 nM) with S-2366 (500 μM) and in HBS-BSA buffer. All proteins were incubated with aZ-mAb-4, -6, or -7 (400 nM) for 1 h at 37 °C. All samples were measured for 30 min except for HGFA, which was measured for 60 min.

**Zymogen matriptase crystallization and structure model construction**

The active-site–mutated zymogen-locked form of matriptase was used for crystallization, containing three mutations: R614A, N772Q, and S805A (zSPD-S805A; Fig. 1D). Initial crystallization conditions were screened using commercial kits (Clear Strategy I, Clear Strategy II, Structure Screen I + II, and JCSG-plus). 0.2 μl of protein was mixed with 0.2 or 0.1 μl of mother liquid using a Mosquito crystallization robot by sitting-drop vapor diffusion at 20 °C. The diffracted crystal was obtained in 0.1 M MES, pH 6.5, 2.0 M ammonium sulfate. For X-ray data collection, the crystal was transferred to a cryoprotectant solution containing 20% glycerol, 0.1 M MES, pH 6.5, 2.0 M ammonium sulfate and flash-frozen in liquid nitrogen. The diffraction data set was collected at a Micro-focus beam line in the European Synchrotron Radiation Facility. The data were processed to a resolution of 2.5 Å using XDS. Data collection statistics and cell parameters are summarized in Table 3. The diffraction data were indexed to the space group P 3(1)21 with a corresponding Matthews coefficient indicating that the crystals contained three molecules in the asymmetric unit (87 kDa). The first two molecules were found by molecular replacement using BALBES and the active matriptase catalytic domain as search model (PDB entry 4ISN) with initial $R_{free}$ of 0.42. Based on the phase from the first two molecules, the missing third molecule was found by Molrep using fast rotation function and phased translation function. The model was built using manual building in COOT and refined in Phenix by applying NCS and TLS throughout the refinement cycles. The final structure was refined to the $R$ and $R_{free}$ values of 0.214 and 0.263 and a tight stereochemistry with root mean square deviations of bond lengths and bond angles of 0.02 Å and 2.0°, respectively.

**Matriptase activity in cell lysates**

HEK293 cells were transiently transfected with empty expression vector (mock) or expression vectors encoding matriptase together with WT HAI-2 or mutant HAI-2 C47F, respectively, using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. The constructs encoding full-length human WT matriptase and HAI-2 were inserted into pcDNA3.1 plasmid vectors (Thermo Fisher Scientific) as described previously by Nonboe et al. (29). HAI-2 was co-expressed to ensure stable matriptase expression, and the HAI-2 C47F mutant was included to allow measurable matriptase activity. Cells were grown for ~48 h before they were lysed on ice in 150 μl of lysis buffer (PBS including 1% Triton X-100 and 0.5% sodium deoxycholate). Protein extracts were prepared by centrifuging the lysates at 20,000 × g for 20 min at 4 °C to remove cell debris. 5 μg of antibody (anti-uPA, aZ-mAb-6, aZ-mAb-7, or no antibody) was added to 60 μl of the protein extracts and incubated at room temperature for 1 h.
Samples were subsequently added to substrate S-2288 in HBS including 0.1% BSA to a final concentration of 300 μM in a total reaction volume of 200 μl. Absorbance was measured in a plate reader at 405 nm continuously every 5 min for 5 h, and the mean velocity of the substrate reaction (in milli-absorbance units/min) was calculated.

**Matriptase-mediated pro-HGF cleavage**

zSPD (200 nm) or acSPD (1 nm) was preincubated with either 20 times the K₅₀ of aZ-mAb-6 or -7 (420 and 180 nm, respectively), 420 nm anti-uPA, or 5 μM biotin-RQRR-CMK for 1 h at 37 °C in PBS, pH 7.4, including 0.1% Triton X-100. Recombinant human pro-HGF (75 ng, R&D Systems) was added, and the samples were incubated for 4.5 h at 37 °C before the addition of reducing loading dye (including 200 μM DTT). Samples containing antibodies alone were prepared in parallel. The Western blotting was performed as described by Nonboe et al. (2019) using anti-HGF antibody (R&D Systems, AF-294-SP) and Matriptase-mediated pro-HGF cleavage

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**Inhibition of zymogen matriptase activity**

1. Bugge, T. H., Antalis, T. M., and Wu, Q. (2009) Type II transmembrane serine proteases. *J. Biol. Chem.* 284, 23177–23181 CrossRef Medline

2. List, K., Haudenschild, C. C., Szabo, R., Chen, W., Wahl, S. M., Swaim, W., Engelholm, L. H., Behrendt, N., and Bugge, T. H. (2002) Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 21, 3765–3779 CrossRef Medline

3. List, K. (2009) Matriptase: a culprit in cancer? *Future Oncol.* 5, 97–104 CrossRef Medline

4. Basel-Vanagaite, L., Attia, R., Ishida-Yamamoto, A., Rainstein, L., Ben Amiat, D., Lurie, R., Pasmanik-Chor, M., Indelman, M., Zvulunov, A., Saban, S., Magal, N., Sprecher, E., and Shohat, M. (2007) Autosomal recessive ichthyosis with hypotrichosis caused by a mutation in ST14, encoding human matriptase. *Am. J. Hum. Genet.* 80, 467–477 CrossRef Medline

5. Lee, S. L., Dickson, R. B., and Lin, C. Y. (2000) Activation of hepatocyte growth factor factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J. Biol. Chem.* 275, 36720–36725 CrossRef Medline

6. Ustach, C. V., Huang, W., Conley-LaComb, M. K., Lin, C. Y., Che, M., Abrams, J., and Kim, H. R. (2010) A novel signaling axis of matriptase/PGDF-D/ss-PDGF in human prostate cancer. *Cancer Res.* 70, 9631–9640 CrossRef Medline

7. Sales, K. U., Friis, S., Konkel, J. E., Godiksen, S., Hatakayama, M., Hansen, K. K., Rogatto, S. R., Szabo, R., Vogel, L. K., Chen, W., Gutkind, J. S., and Bugge, T. H. (2015) Non-hematopoietic PAR-2 is essential for matriptase-driven pre-malignant progression and potentiation of ras-mediated squamous cell carcinomaogenesis. *Oncogene* 34, 346–356 CrossRef Medline

8. Miller, G. S., and List, K. (2013) The matriptase-prostasin proteolytic cascade in epithelial development and pathology. *Cell Tissue Res.* 351, 245–253 CrossRef Medline

9. Wu, C. J., Feng, X., Lu, M., Morimura, S., and Udey, M. C. (2017) Matriptase-mediated cleavage of EpCAM destabilizes claudins and dysregulates intestinal epithelial homeostasis. *J. Clin. Invest.* 127, 623–634 CrossRef Medline

10. Szabo, R., Hobson, J. P., List, K., Molinolo, A., Lin, C. Y., and Bugge, T. H. (2008) Potent inhibition and global co-localization implicate the transmembrane Kunitz-type serine protease inhibitor hepatocyte growth factor activator inhibitor-2 in the regulation of epithelial matriptase activity. *J. Biol. Chem.* 283, 29495–29504 CrossRef Medline

11. Szabo, R., Hobson, J. P., Christoph, K., Kosa, P., List, K., and Bugge, T. H. (2009) Regulation of cell surface protease matriptase by HA12 is essential for placental development, neural tube closure and embryonic survival in mice. *Development* 136, 2653–2663 CrossRef Medline

12. Szabo, R., Molinolo, A., List, K., and Bugge, T. H. (2007) Matriptase inhibition by hepatocyte growth factor activator inhibitor-1 is essential for placental development. *Oncogene* 26, 1546–1556 CrossRef Medline

13. Sales, K. U., Friis, S., Absuleme, L., Moutsopoulos, N. M., and Bugge, T. H. (2015) Matriptase promotes inflammatory cell accumulation and progression of established epidermal tumors. *Oncogene* 34, 4664–4672 CrossRef Medline

14. List, K., Szabo, R., Molinolo, A., Suriarunpong, V., Redeye, V., Murdock, T., Burke, B., Nielsen, B. S., Gutkind, J. S., and Bugge, T. H. (2005) Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev.* 19, 1394–1395 CrossRef Medline

15. Tanabe, L. M., and List, K. (2017) The role of type II transmembrane serine protease mediated signaling in cancer. *FEBS J.* 284, 1421–1436 CrossRef Medline

16. Huber, R., and Bode, W. (1978) Structural basis of the activation and action of trypsin. *Acc. Chem. Res.* 11, 114–122 CrossRef Medline

17. Wang, D., Bode, W., and Huber, R. (1985) Bovine chymotrypsinogen A. X-ray crystal structure analysis and refinement of a new crystal form at 1.8 Å resolution. *J. Mol. Biol.* 185, 595–624 CrossRef Medline

18. Berg, J. M., Tymoczko, J. L., and Stryer, L. (2012) *Biochemistry*, 7th Ed. W.H. Freeman & Co., New York

19. Madison, E. L., Kobe, A., Gething, M. J., Sambrook, J. F., and Goldsmith, E. J. (1993) Converting tissue plasminogen activator to a zymogen: a regulatory triad of Asp-His-Ser. *Science* 262, 419–421 CrossRef Medline

20. Inouye, K., Yasumoto, M., Tsuzuki, S., Mochida, S., and Fushiki, T. (2010) The optimal activity of a pseudozymogen form of recombinant matriptase under the mildly acidic pH and low ionic strength conditions. *J. Biochem.* 147, 485–492 CrossRef Medline

21. Oberst, M. D., Williams, C. A., Dickson, R. B., Johnson, M. D., and Lin, C.-Y. (2003) The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J. Biol. Chem.* 278, 26773–26779 CrossRef Medline

22. Tseng, I. C., Xu, H., Chou, F. B., Li, G., Vazzano, A. P., Kao, J. P. Y., Johnson, M. D., and Lin, C. Y. (2010) Matriptase activation, an early cellular response to acidosis. *J. Biol. Chem.* 285, 3261–3270 CrossRef Medline

23. Godiksen, S., Soendergaard, C., Friis, S., Jensen, K. J., Bornholdt, I., Sales, K. U., Huang, M., Bugge, T. H., and Vogel, L. K. (2013) Detection of active matriptase using a biotinylated chloromethyl ketone peptide. *PLoS One* 8, e71146 CrossRef Medline

24. Friis, S., Tadeo, D., Le-Gall, S. M., Jürgensen, H. J., Sales, K. U., Camerer, E., and Bugge, T. H. (2017) Matriptase zymogen supports epithelial development, homeostasis and regeneration. *BMC Biol.* 15, 46 CrossRef Medline

25. Gray, K., Elghadban, S., Thongyoo, P., Owen, K. A., Szabo, R., Bugge, T. H., Tate, E. W., Leatherbarrow, R. J., and Ellis, V. (2014) Potent and specific inhibition of the biological activity of the type-II transmembrane serine protease matriptase by the cyclic micrornecoin MCoTI-II. *Thromb. Haemost.* 112, 402–411 CrossRef Medline

26. Colombo, E., Désilets, A., Duchêne, D., Chagnon, F., Najmanovich, R., Leduc, R., and Marsault, E. (2012) Design and synthesis of potent, selective inhibitors of matriptase. *ACS Med. Chem. Lett.* 3, 530–534 CrossRef Medline

27. Schneider, E. L., Lee, M. S., Baharuddin, A., Goetz, D. H., Farady, C. J., Ward, M., Wang, C. I., and Craik, C. S. (2012) A reverse binding motif that
Inhibition of zymogen matriptase activity

contributes to specific protease inhibition by antibodies. J. Mol. Biol. 415, 699–715 CrossRefMedline

28. Xu, Z., Chen, Y. W., Battu, A., Wilder, P., Weber, D., Yu, W., Mackerell, A. D., Chen, L. M., Chai, K. X., Johnson, M. D., and Lin, C. Y. (2011) Targeting zymogen activation to control the matriptase-prostasin proteolytic cascade. J. Med. Chem. 54, 7567–7578 CrossRefMedline

29. Nonboe, A. W., Krigslund, O., Soendergaard, C., Skovbjerg, S., Friis, S., Andersen, M. N., Ellis, V., Kawaguchi, M., Kataoka, H., Bugge, T. H., and Vogel, L. K. (2017) HAI-2 stabilizes, inhibits and regulates SEA-cleavage-dependent secretory transport of matriptase. Traffic 18, 378–391 CrossRefMedline

30. Hong, Z., De Meulemeester, L., Jacobi, A., Pedersen, J. S., Morth, J. P., Andreasen, P. A., and Jensen, J. K. (2016) Crystal structure of a two-domain fragment of hepatocyte growth factor activator inhibitor-1: functional interactions between the Kunitz-type inhibitor domain-1 and the neighboring polycystic kidney disease-like domain. J. Biol. Chem. 291, 14340–14355 CrossRefMedline

31. Zhao, B., Yuan, C., Li, R., Qu, D., Huang, M., and Ngo, J. C. K. (2013) Crystal structures of matriptase in complex with its inhibitor hepatocyte growth factor activator inhibitor-1. J. Biol. Chem. 288, 11155–11164 CrossRefMedline

32. Friis, S., Uzzun Sales, K., Godiksen, S., Peters, D. E., Lin, C.-Y., Vogel, L. K., and Bugge, T. H. (2013) A matriptase-prostasin reciprocal zymogen activation complex with unique features: prostasin as a non-enzymatic co-factor for matriptase activation. J. Biol. Chem. 288, 19028–19039 CrossRefMedline

33. Takeuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R., and Craik, C. S. (2000) Cellular Localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J. Biol. Chem. 275, 26333–26342 CrossRefMedline

34. Pozzi, N., Chen, Z., Zapata, F., Niu, W., Barranco-Medina, S., Pelc, L. A., and Di Cera, E. (2013) Autoactivation of thrombin precursors. J. Biol. Chem. 288, 11601–11610 CrossRefMedline

35. Kataoka, H., Kawaguchi, M., Fukushima, T., and Shimomura, T. (2018) Hepatocyte growth factor activator inhibitors (HAI-1 and HAI-2): emerging key players in epithelial integrity and cancer. Pathol. Int. 68, 145–158 CrossRefMedline

36. Farady, C. J., and Craik, C. S. (2010) Mechanisms of macromolecular protease inhibitors. ChemBioChem 11, 2341–2346 CrossRefMedline

37. Köhler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497 CrossRefMedline

38. Reading, C. L. (1982) Theory and methods for immunization in culture and monoclonal antibody production. J. Immunol. Methods 53, 261–291 CrossRefMedline

39. Copeland, R. A. (2000) Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis, 2nd Ed., Wiley-VCH, New York