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REVIEW ARTICLE

Structural and mechanistic insight into how antibodies inhibit serine proteases

Rajkumar GANESAN*, Charles EIGENBROT† and Daniel KIRCHHOFER*[1]

*Department of Protein Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, U.S.A., and †Department of Structural Biology, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, U.S.A.

Antibodies display great versatility in protein interactions and have become important therapeutic agents for a variety of human diseases. Their ability to discriminate between highly conserved sequences could be of great use for therapeutic approaches that target proteases, for which structural features are conserved among family members. Recent crystal structures of antibody–protease complexes provide exciting insight into the variety of ways antibodies can interfere with the catalytic machinery of serine proteases. The studies revealed the molecular details of two fundamental mechanisms by which antibodies inhibit catalysis of trypsin-like serine proteases, exemplified by hepatocyte growth factor activator and MT-SP1 (matriptase). Enzyme kinetics defines both mechanisms as competitive inhibition systems, yet, on the molecular level, they involve distinct structural elements of the active-site region. In the steric hindrance mechanism, the antibody binds to protruding surface loops and inserts one or two CDR (complementarity-determining region) loops into the enzyme's substrate-binding cleft, which results in obstruction of substrate access. In the allosteric inhibition mechanism the antibody binds outside the active site at the periphery of the substrate-binding cleft and, mediated through a conformational change of a surface loop, imposes structural changes at important substrate interaction sites resulting in impaired catalysis. At the centre of this allosteric mechanism is the 99-loop, which is sandwiched between the substrate and the antibody-binding sites and serves as a mobile conduit between these sites. These findings provide comprehensive structural and functional insight into the molecular versatility of antibodies for interfering with the catalytic machinery of proteases.

Key words: allosteric regulation, antibody, inhibition mechanism, phage display, pseudo-protease, serine protease.

INTRODUCTION

Proteases hydrolyse peptide bonds of protein and peptide substrates, an activity which is fundamental to many aspects of human physiology including blood coagulation, fibrinolysis, food digestion, complement activation, tissue regeneration and blood pressure regulation. In these processes the temporal and spatial activity of proteases is regulated at the transcriptional as well as post-translational levels, e.g. by zymogen activation, by cofactors and by inhibitors. Many diseases are associated with dysregulated protease activity. For instance, too much activity by trypsin-like coagulation proteases can lead to venous thrombosis, stroke, myocardial infarction or disseminated intravascular coagulation. Thus the therapeutic potential of targeting proteases is significant. Many specific as well as relatively non-specific protease inhibitors are currently used in disease management ranging from cardiovascular disease [e.g. anticoagulants and ACE (angiotensin-converting enzyme) inhibitors] to viral infection, diabetes and cancer [1].

Specificity is generally a desired property for inhibiting protease activity, but is often difficult to achieve. This is due to the relatively conserved active-site topologies among members of the same protease family, which presents a challenge for developing selective active-site inhibitors. With respect to physiological processes, the conservation of active-site topologies imparts an increased regulation potential as most natural protease inhibitors can inhibit the activities of more than one protease. Antibodies, on the other hand, have exquisite specificity, excellent pharmacokinetic properties and are widely used as therapeutic agents [2]. Therefore it is logical to consider antibodies as potential modifiers of therapeutically relevant proteases [3]. Of course, the realm of proteases amenable to normal antibodies currently does not include the central nervous system or the intracellular compartment. However, since approximately half of all proteases in the human genome are extracellular [4], the number of proteases potentially targetable by antibodies seems substantial. In the present review we discuss the recently obtained structural insights into the mechanisms by which antibodies interfere with the catalytic activity of serine proteases.

ANTIBODIES AS THERAPEUTICS

Antibodies (or immunoglobulins) are produced by immune cells in response to substances (antigens) that are recognized as foreign by the human body. The five different classes of antibodies (IgD, IgA, IgM, IgE and IgG) perform different functions in the immune system. IgGs are the most abundant immunoglobulins in the blood and are the dominant class among approved therapeutic antibodies. The structure of an IgG can be described as a 'Y'-shaped molecule comprising two Fabs, which are linked via a flexible region (hinge) to a complement-binding fragment (Fc) region (Figure 1A). Antibodies are usually composed of two identical light chains (L) and two identical heavy chains (H) which

Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; CDR, complementarity-determining region; HGF, hepatocyte growth factor; HGFA, HGF activator; MSP, macrophage-stimulating protein; PSA, prostate-specific antigen; scFv, single-chain variable domain fragment; Shh, Sonic hedgehog; uPA, urokinase-type plasminogen activator; ZPI, Protein Z-dependent inhibitor.

[1] To whom correspondence should be addressed (email dak@gene.com).

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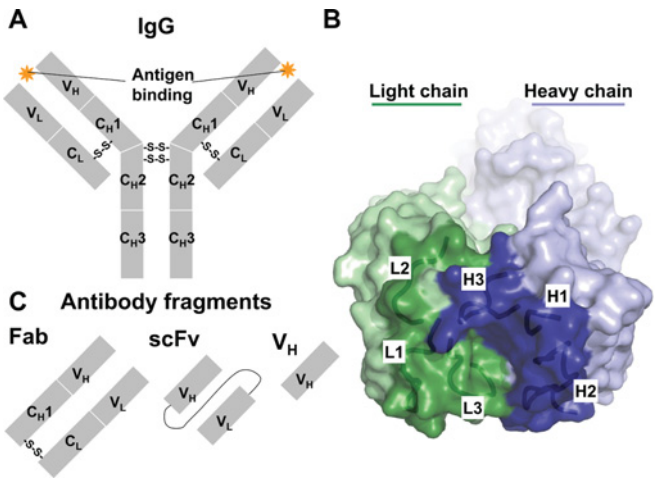

Figure 1 Domain architecture of antibodies and antibody fragments

(**A**) Conventional antibody (IgG class) has: V_H (heavy chain, variable domain), V_L (light chain, variable domain), C_H1, 2, 3 (heavy chain, constant domains) and C_L (light chain, constant domain). The antigen-binding site is formed by the CDR loops of the V_H and V_L domains. (**B**) Surface representation of a Fab with heavy chain (blue) and light chain (green). The CDRs are shown in darker shades (CDR H1–H3 blue, CDR L1–L3 green). (**C**) Antibody fragments: Fab, single-chain-Fv (scFv) and V_H domain.

are linked together by disulfide bonds. Heavy chains contain a variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3), whereas the light chains contain a variable domain (V_L domain) and a single constant domain (C_L domain). The variable domains encompass three regions of sequence hypervariability called CDRs (complementarity-determining regions) (Figure 1B). They differ in length and sequence between different antibodies and are responsible for antigen specificity (recognition) and affinity (binding). Although the six CDR sequences of an antibody act co-operatively, CDR-H3 is the most diverse region in length and sequence and is often the major contributor to antigen binding. The antibody contact region on the antigen is referred to as the epitope, whereas the corresponding region on the antibody is referred to as the paratope [5]. Antibodies constitute a large class of biopharmaceuticals, with 24 products approved for use in humans, mostly for oncology and immunology indications. There are more than 200 antibodies in different stages of clinical development [5–9].

ANTIBODY FRAGMENTS AS THERAPEUTICS

In addition to full-length antibodies, several types of antibody fragments are also being developed as potential therapeutics (Figure 1C). Fabs and scFv (single-chain variable domain fragments, heterodimers of V_H and V_L domains connected by a flexible linker to circumvent the problems of stability of non-covalent Fv) are probably the most studied antibody fragments (Figure 1C). In fact, there are already three FDA (Food and Drug Administration) approved drugs which are Fab fragments: Abciximab (ReoPro®) is a chimaeric Fab which inhibits platelet activation by blocking the platelet glycoprotein IIb/IIIa receptor and was the first to be approved in 1994 [10,11], followed by ranibizumab (Lucentis®), a humanized VEGF (vascular endothelial growth factor)-specific Fab for treatment of neovascular (wet) AMD (age-related macular degeneration) [12,13] and Certolizumab pegol (CIMZIA®), a PEGylated anti-TNFα (tumour necrosis factor α) Fab for the treatment of Crohn's disease [14]. In addition, there are many clinical trials with molecules such as pexelizumab, a humanized scFv that is in phase II/III testing for patients undergoing graft surgery for coronary artery bypass [15,16].

Efforts to further reduce the size of an antibody fragment yielded single domains, such as the isolated V_H domains, which constitute the smallest functional unit for antigen binding (Figure 1C). Ward et al. [17] screened a repertoire of isolated murine V_H domains, which led to the identification of binders against lysozyme. This was the first study to show that antibody fragments, owing to their small size, could be useful to target cryptic epitopes, such as an enzyme active-site cleft. To escape the host defence mechanism, many pathogenic viruses have evolved narrow cavities or canyons in their surface antigens. These structural elements are critical for binding to host receptors, but are generally poorly accessible to full-length antibodies. Owing to limited diversity of the CDR-loop lengths, full-length antibodies mostly recognize flat or concave protein epitopes [18,19], whereas V_H domains with long CDR-H3 loops have a prolate shape and are better suited to target enzyme active-site clefts. Characterization of natural heavy-chain antibodies, such as V_HH of camelids and V-NARs of sharks, allowed a critical insight into the structural basis of their autonomous stability and solubility. Thus it became possible to engineer autonomous versions of conventional V_H domains with favourable biophysical properties. For instance, to circumvent poor solubility arising from exposure of a part of V_H usually buried in its interface with a V_L domain, Barthelemy et al. [20] introduced a guided molecular evolution technique to mutate a human V_H into a stabilized, reversibly refolding, better expressing and autonomous single-domain antibody fragment.

ANTIBODIES AS PROTEASE INHIBITORS

Naturally occurring protease inhibitors interfere with catalysis by presenting a protruding reactive-site loop, which interacts with the concave substrate-binding cleft in a substrate-like manner. Comparison of the reactive-site loop conformations of 18 convergently evolved inhibitor family members showed that they are virtually superimposable [21], indicating that natural inhibitors interact with the active-site cleft in a canonical (substrate-like) manner. Drug discovery efforts to re-engineer physiological protease inhibitors were made by the application of phage-display technology [22,23]. An example of this approach was the engineering of the Kunitz domains inhibitors against proteases [24–27]. With a prolate shape similar to physiological protease inhibitors, camelid heavy-chain antibodies, which lack any light chain, are well-suited to insert a loop into the cleft. Structural studies with the hydrolase lysozyme demonstrated that the relatively long CDR-H3 loop gives the V_H domain a convex shape optimal for cleft insertion [28]. This shape complementarity is rare in conventional antibodies and it was found that the available conventional anti-lysozyme antibodies did not bind into the cleft, were non-blocking and did not act as inhibitors. In addition, although H3 loops are of variable lengths, their average length (nine residues for mouse and 12 residues for human sequences [29]) might be insufficient for canonical binding. This raised the intriguing question of how do conventional antibodies interfere with the catalytic machinery? In what follows, we focus on antibodies that bind directly to a protease domain and do not discuss antibodies which block protease activity by interfering with regulators, such as inducers of zymogen activation [30] or cofactors [31], or which interfere with cell-surface binding, which are also effective anti-protease approaches.

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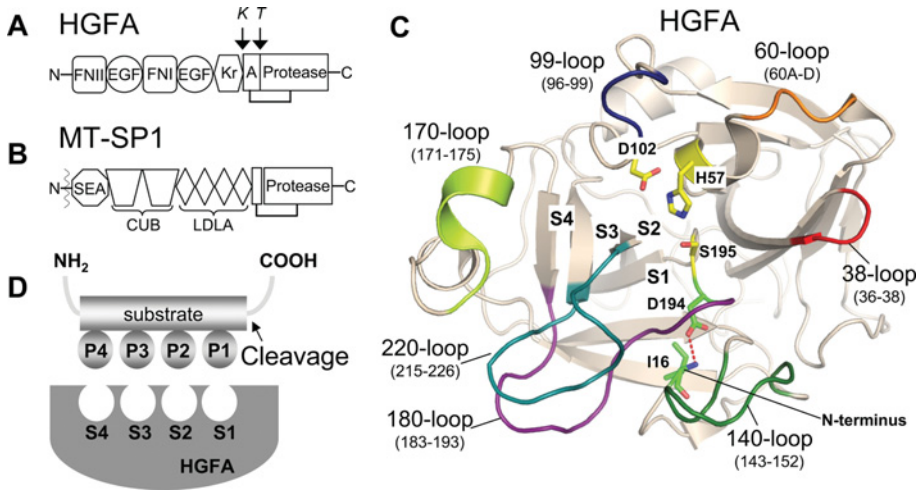

Figure 2 HGFA and MT-SP1

(**A**) The domain architecture of HGFA. HGFA is composed of a fibronectin type-2 domain (FNII), a fibronectin type-1 domain (FNI), two epidermal growth factor domains (EGF), a Kringle domain (Kr) and a serine protease domain (protease). Activation of pro-HGFA occurs by proteolytic cleavage (black arrows) by thrombin (T) and kallikrein (K). The HGFA A-chain (A) is linked to the protease domain by a disulfide bond. (**B**) The domain architecture of MT-SP1. MT-SP1 is composed of a cytoplasmic domain, transmembrane region, a SEA domain, two CUB domains (CUB), four LDL (low-density lipoprotein) receptor type-A domains (LDLA) and a serine protease domain (protease). (**C**) HGFA protease domain (PDB code 1YC0) with coloured substrate-specificity determining loops and substrate subsites S1–S4. The catalytic triad (stick representation, yellow) Asp^{102}–His^{57}–Ser^{195} is indicated. The N-terminus insertion after zymogen activation is accompanied with the formation of a salt bridge (red dotted line) between the primary amine of the Ile^{16} and Asp^{194} (both are indicated in stick representation, green). (**D**) Cartoon of important substrate residues (P1–P4) interacting with protease subsites (S1–S4) according to the nomenclature described by Schechter and Berger [61]. The P1 residue of HGFA substrates is always an arginine and for MT-SP1 substrates it is either arginine or lysine. Single letter amino acid codes are used in the Figure.

Production of antibodies against serine proteases dates back to more than half-a-century ago, when Verwilghen et al. [32] first described an anti-thrombin antibody of equine origin. Subsequently, several antibodies that bind to protease domains and block catalysis have been generated [33–36], and in a few cases the inhibition mechanisms have been described biochemically and enzyme kinetically [37]. Neutralizing antibodies against several trypsin-like serine proteases have been reported, which include uPA (urokinase-type plasminogen activator) [38–40], thrombin [41], coagulation Factor VIIa [42], MT-SP1 (also known as matriptase) [43,44], hepsin [45] and NS3 protease from hepatitis C virus [46]. In addition to full-length antibodies, identification and characterization of antibody fragments against serine proteases have also been reported [47,48]. Biochemical studies did provide information about the antibody-binding epitope on the proteases apart from providing hints on the mode of inhibition. The most common mode of inhibition is a competitive inhibition mechanism [47], but in some instances a mixed-type inhibition [42] has also been observed for antibody inhibitors against serine proteases. Epitope mapping was commonly performed by mutagenesis experiments [39], sometimes complemented by binding experiments in the presence of an active-site inhibitor [42,46,47,49]. For instance, using a panel of 55 surface mutants of recombinant thrombin, Colwell et al. [41] showed that the epitope for an anti-thrombin IgG was most likely to be located in exosite II. However, a more detailed understanding of the underlying molecular mechanism was hampered by the absence of structural information. Several structures of antibody Fabs in complex with trypsin-like serine proteases (Clan PA, family S1) provide unprecedented new insight into the molecular details by which antibodies interfere with the catalytic machinery [50–52]. Although the antibodies act as competitive inhibitors based on enzyme kinetic analysis, the structures define two fundamentally different mechanisms utilized by antibodies.

ANTIBODIES AGAINST THE TRYPSIN-LIKE SERINE PROTEASES HGFA [HGF (HEPATOCYTE GROWTH FACTOR) ACTIVATOR] AND MT-SP1

The first mechanism was identified by Wu et al. [52] and Farady et al. [51] using antibodies against two different proteases of the same family (S1) of serine proteases, HGFA and MT-SP1 (also known as matriptase) (Figures 2A and 2B). HGFA has restrictive substrate specificity as it is known to cleave only two macromolecular substrates, pro-HGF [53] and pro-MSP (macrophage-stimulating protein) [54]. It is inhibited by multiple biological inhibitors including the Kunitz domain inhibitor HAI-1 (HGFA inhibitor-1) [55–57]. HGFA has the same domain architecture (Figure 2A) as coagulation Factor XII but, during blood coagulation, is processed to a short serum form consisting mainly of the protease domain [53]. HGFA is involved in tissue regeneration and tumorigenesis via pro-HGF processing and ensuing activation of the HGF/Met signalling pathway [53]. MT-SP1 is a member of the type II transmembrane serine protease family and is involved in terminal epithelial differentiation and skin barrier function [58]. Overexpression of MT-SP1 is associated with tumour initiation and progression, possibly mediated by some of its identified substrates, such as pro-HGF, pro-uPA and PAR-2 [58]. The structural studies discussed below were carried out with the protease domains of MT-SP1 and HGFA (Figures 2A and 2B) expressed in *Escherichia coli* and insect cells respectively.

Previously determined X-ray structures of both proteases in complex with Kunitz domain inhibitors [59,60] show the typical double β-barrel arrangement of the peptidase domain and the canyon-like substrate-binding cleft, which is somewhat deeper in matriptase than in HGFA. Some important features of the active site region are illustrated in Figure 2(C) using HGFA as an example. Specific amino acid positions will use the chymotrypsinogen numbering scheme to ease reference to

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the large number of related proteins, and we will employ the nomenclature of Schechter and Berger [61] in describing specific sites of protease–substrate (or antibody) interactions. Figure 2(C) shows the His[57]-Asp[102]-Ser[195] catalytic triad and the inferred positions of substrate subsites S1–S4 interacting with substrate residues P1–P4 (Figures 2C and 2D). The principal determinant of substrate preference is the S1-specificity pocket. The Asp[189] residue in HGFA and MT-SP1 at the bottom of the S1 pocket confers a strong preference for substrates having an arginine or lysine as their P1 residue, consistent with the P1 residues found in their known macromolecular substrates. The loops surrounding the active-site cleft help determine substrate and inhibitor specificities (Figure 2C). Accordingly, these loops have relatively low sequence homology. Although there is significant length diversity for these loops among family members, for HGFA and matriptase only the long 60s-loop in matriptase is noteworthy, and it is similar to the loop length found in thrombin.

MECHANISM 1: STERIC HINDRANCE OF SUBSTRATE ACCESS TO THE CATALYTIC CLEFT

Anti-HGFA antibody (Fab58)

The anti-HGFA antibody (Fab58) and anti-MT-SP1 antibody (FabE2) are potent inhibitors derived from phage-displayed Fab and scFv libraries respectively [44,51,52]. Both antibodies are competitive inhibitors and can completely inhibit proteolysis of small synthetic, as well as macromolecular, substrates. Yet competition binding studies with small inhibitors that only occupy the S1 pocket hinted at somewhat different inhibition mechanisms applied by the two antibodies: the FabE2 interaction was disrupted by S1 occupancy, whereas the Fab58 interaction was not [51,52]. The crystal structures of the Fab58–HGFA complex at 3.5 Å (1 Å = 0.1 nm) (PDB code 2R0K) and FabE2–MT-SP1 complex at 2.17 Å (PDB code 3BN9) provided the answers. Most Fab58 contacts were with residues of substrate-specificity determining loops (60- and 99-loops) that form one side of the canyon-like HGFA substrate-binding cleft, with additional contacts on the opposite side (170-, 220- and 140-loops) (Figures 3A and 3B). Inhibition of catalysis was caused by insertion of the CDR-H2- and H1-loops into the cleft to occupy the S2 and S3/S4 subsites of HGFA, which are critical for interaction with P2 and P3 residues of substrates (Figure 3B). The side chain of Asn[53] of the H2 loop occupies the same position as the P2-leucine side chain of KQLR-chloromethyl ketone inhibitor, which mimics the interaction of the substrate (pro-HGF) residues KQLR (P4–P1) (Figure 3C). In addition, Thr[30], Gly[31] and Ser[32] of the H1 loop occupy the S3/S4 subsites. However, the CDR loops did not reach the S1 pocket, which remained empty. This explains why Fab58 could bind to HGFA in the presence of benzamidine, which occupies the S1 pocket. In contrast with camelid heavy-chain antibodies, the H3-loop of Fab58 does not insert into the cleft at all, but rather partners with the L3-loop to embrace Phe[97] at the ridge of the protruding 99-loop (Figure 3B). Thus, compared with the interaction of convex camelid antibodies with the concave lysozyme enzyme cleft [28], the interaction of Fab58 with HGFA is inverted, as Fab58 uses the concave V_H/V_L cleft to interact with a convex structural feature of the enzyme, i.e. the protruding 99-loop.

Anti-MT-SP1 antibody (FabE2)

The structural epitope of FabE2 is similar to Fab58 in that FabE2 binds to surface loops on both sides of the canyon-like cleft (60-, 99-, 140-, 170-, 180- and 220-loop) (Figures 3D and 3E). However, unlike Fab58, it inserts a very long CDR-H3 loop into the cleft, to occupy the subsites S1–S4 (Figure 3E). The S1 cavity is partially occupied by the Arg[100b] side chain of FabE2 (Figure 3F), which accounts for the finding that MT-SP1 could cleave CDR-H3, albeit with very low efficiency. The long CDR-H3 loop of FabE2 contributes more than two-thirds of the total binding area and in some aspects, mimics the protruding convex loops of V_H domain binders of lysozyme. The FabE2 CDR-H3 residues occupy several subsites: the Arg[100c] at the S1′ subsite, Gly[100d] at the S2 subsite and Pro[100e] in the S3/S4 subsites. An interesting feature of FabE2 binding is that, even though H3 loop residues occupy substrate-binding subsites, they do not bind in a strictly canonical manner, since they approach the cleft in the reverse order (from C-terminus to N-terminus) without involving any inter-main-chain interaction that is typical for canonical inhibitors. Comparison of the known crystal structures of MT-SP1 indicate that the 99-loop can adopt different conformations [51,59,62]. The 99-loop conformation in the BPTI (bovine pancreatic trypsin inhibitor)–MT-SP1 complex [59] provides an 'open' S2 pocket for optimal P2–S2 interaction. In contrast, the conformation of the 99-loop in the FabE2–MT-SP1 structure results in a 'closed' S2 pocket that would cause a steric clash with the P2 residue of BPTI as well as with the P2-leucine of the modelled KQLR-chloromethyl ketone inhibitor (Figure 3F). Therefore it appears that the FabE2 specifically binds to the MT-SP1 conformation with a 'closed' S2 subsite. Moreover, Arg[100b] occupies the S1 pocket, but approaches at a different angle compared with P1-lysine of BPTI and the modelled P1-arginine of the KQLR inhibitor.

Although the details of the antibody–protease interaction differ between the discussed complexes, a common feature is that both antibodies (Fab58 and FabE2) use protease surface loops as a 'grip' and utilize CDR loops of the heavy chain for insertion into the canyon-like cleft to occupy important subsites. As illustrated in the animation of the Fab58–HGFA interaction (see Supplementary Movie S1 at http://www.BiochemJ.org/bj/430/bj4300179add.htm) and in the cartoon model in Figure 4(A), the competitive inhibition mechanism is due to the steric hindrance of substrate binding in the active-site region.

MECHANISM 2: ALLOSTERIC INHIBITION

The 'allosteric switch' mechanism

The first structural evidence for an allosteric mechanism came from the Fab75–HGFA complex (PDB code 2R0L), but the mechanistic details remained elusive due to crystal packing interactions [52]. However, subsequent studies with a related anti-HGFA antibody, Fab40, provided the answers. Three different crystal structures unraveled an 'allosteric switch' mechanism and provided the molecular basis for rationalizing the partial competitive inhibition mode determined by enzyme kinetics [50]. The structure of the Fab40–HGFA complex at 2.35 Å resolution (PDB code 3K2U) demonstrates that the antibody does not bind to the active-site region, but interacts with a relatively flat epitope at the periphery of the substrate-binding cleft (Figure 5A). The substrate subsites S1–S4 are not occupied (Figure 5A), and the catalytic triad residues show no major conformational changes. The key feature is a significant conformational change of the 99-loop, which embodies the allosteric switch as seen by comparison with other HGFA structures in which the 99-loop is in a competent conformation (enzymatically active) (Figure 5B). The non-competent (catalytically inactive) conformation of the

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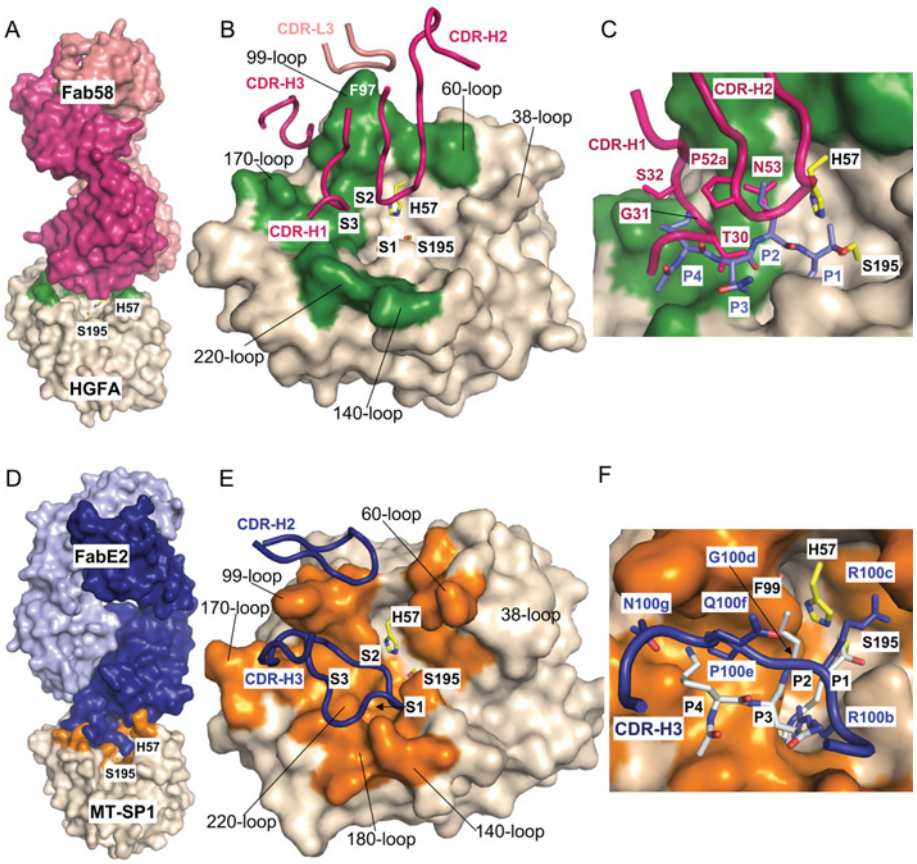

Figure 3 Protease inhibition by steric hindrance of substrate access

(**A**) The Fab58–HGFA complex with Fab light and heavy chains in light and dark pink and the contact region on HGFA in green (4 Å cut-off). Note that Fab58 does not obstruct the S1 pocket, which remains empty. (**B**) Fab58 makes contacts with the 60-, 99-, 140-, 170- and 220-loops and inserts the CDR-H2 and CDR-H1 loops to occupy the S2–S4 subsites. Also indicated are the catalytic residues His^{57} and Ser^{195}. (**C**) Overlay of the Fab58–HGFA structure with the structure of the HGFA–peptidic inhibitor (Ac-KQLR-chloromethyl ketone) complex to illustrate the subsite occupancy by Fab58 CDR loop residues. The sidechain of Asn^{53} (H2 loop) overlaps with the P2-leucine while the CDR-H1 loop residues, Thr^{30}, Gly^{31} and Ser^{32} occupy the S3 and S4 subsites. (**D**) The FabE2–MT-SP1 complex with FabE2 light and heavy chains in light and dark blue and the contact region (4 Å cut-off) on MT-SP1 in orange. (**E**) FabE2 makes contacts with the 60-, 99-, 140-, 170-, 180- and 220-loops and inserts a long CDR-H3 loop to occupy subsites S1–S4, while CDR-H2 embraces the back of the 99-loop. Also indicated are the catalytic residues His^{57} and Ser^{195}. (**F**) The active site of MT-SP1 from the structure of the FabE2–MT-SP1 complex with the modelled KQLR peptide inhibitor. The Arg^{100b} of the CDR-H3 loop of FabE2 inserts into the S1 pocket but at an angle, which is different from a canonical inhibitor. While Arg^{100c} is orientated towards the S1′ site, Gly^{100d} and Pro^{100e} occupy the S2–S4 subsites. Single letter amino acid codes are used in the Figure.

99-loop seen in the Fab40–HGFA complex is stabilized by interactions with the Fab40 CDR-H3 loop. One of these H3 loop residues, Trp^{96}, inserts into a large hydrophobic pocket, which seems a critical feature to lock the 99-loop into the non-competent conformation (Figure 5B). In an attempt to 'turn off the switch', this key residue was deleted (Fab40ΔTrp) and its effect on the 99-loop conformation analysed structurally and functionally. The results clearly established the regulatory function of the 'switch' by showing that the 99-loop in the Fab40ΔTrp–HGFA complex (2.90 Å resolution; PDB code 2WUB) is 'flipped back' to the original competent state commensurate with the reversal of HGFA to an enzymatically competent enzyme. Figure 5(C) summarizes the different conformational states of the 99-loop.

The competitive nature of allosteric inhibition

How does the non-competent 99-loop conformation impair catalysis? The 99-loop contributes to shaping the S2 and S4 subsites and that is where the obstructions arose. The molecular details were revealed in the structure of Fab40ΔTrp–HGFA with the irreversibly bound KQLR peptide (2.70 Å resolution; PDB code 2WUC), which encompasses the P4–P1 sequence of the macromolecular substrate pro-HGF. The 99-loop residues Pro^{99a} and Ser^{99} together with the catalytic His^{57} form the hydrophobic S2 pocket, which is ideally shaped to accommodate a P2-leucine. The implied preference for a P2-leucine is consistent with the occurrence of leucine as a P2 residue in the macromolecular substrates pro-HGF and pro-MSP, as well as in the synthetic para-nitroanilide substrate used for enzymatic assays. The non-competent 99-loop conformation imposed by Fab40 results in the partial collapse of the S2 pocket and the loss of a stabilizing interaction between P4-lysine and the S4 subsite (Supplementary Movie S2 at http://www.BiochemJ.org/bj/430/bj4300179add.htm, also see Figure 7 in Ganesan et al. [50]). These changes lead to non-optimal interactions with P2 and P4 residues of substrate and are the root cause of Fab40-mediated inhibition.

The elucidated allosteric mechanism provides a suitable framework to understand the competitive nature of HGFA inhibition by Fab40. The 99-loop serves as a mobile conduit connecting two allosteric sites, i.e. the substrate subsites S2/S4

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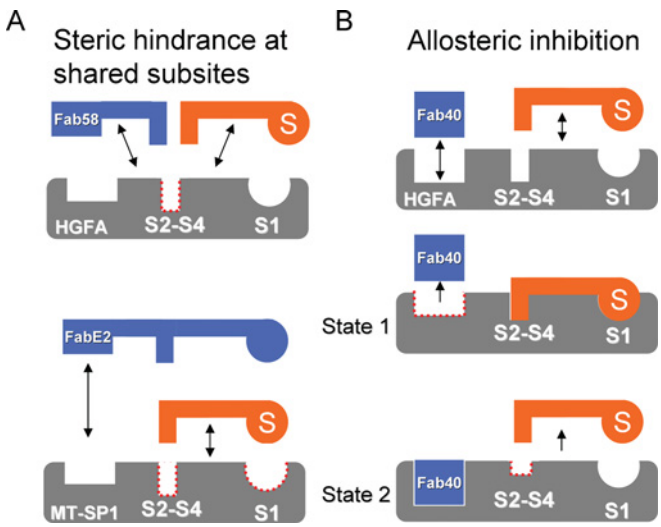

Figure 4 Mechanisms of protease inhibition by antibodies

(**A**) In the steric hindrance mechanism of protease inhibition, an antibody uses its CDR loops to occupy the substrate (S)-binding subsites, i.e. subsites S2–S4 by Fab58 and subsites S1–S4 by FabE2. The subsites affected by antibody binding are indicated by red dotted lines. (**B**) The 99-loop is sandwiched between the substrate-binding site and the Fab40-binding site. In the substrate-bound form HGFA (state 1), the residues of the 99-loop interact with substrate and the conformation of the 99-loop is incompatible with Fab40 binding. In contrast, due to reorganization of the subsites S2–S4, the Fab40-bound state of HGFA (state 2) is incompatible with substrate binding (red dotted lines).

and the antibody-binding site. Both antibody and substrate can apply forces on the 99-loop, albeit from opposite directions resulting in perturbed interactions on either side (Supplementary Movie S2). Thus the Fab40 inhibition mechanism exemplifies a specific case of competitive inhibition systems outlined by Segel [63] and is schematically shown in Figure 4(B). In the substrate-bound form of HGFA (state 1), the residues of the 99-loop are engaged in interaction with the substrate. In this state, the conformation of the 99-loop is incompatible with Fab40 binding, whereas in the Fab40-bound state of HGFA (state 2), the 99-loop conformation is incompatible with substrate binding. The model accounts for the competitive nature of HGFA inhibition, in that an increase of substrate concentration will shift the equilibrium to the competent state (state 1) allowing catalysis to proceed. In contrast, Fab58 and FabE2 act by classic steric hindrance of substrate access to the active-site region by occupying important subsites with their CDR loops (Figure 4A).

EXOSITES AS REGULATORS OF CATALYTIC ACTIVITY

Allostery is a widespread mechanism for modulating protein activity and particularly for regulating catalytic activity of proteases [64]. In the S1 family of serine proteases we find many examples of allosteric regulation, such as the PDZ domain of the bacterial protease DegS [65,66], calcium, sodium and cofactors for various coagulation factors [67–69] and N-terminal peptide insertion into the activation pocket for trypsin-like proteases [70]. The allosteric switch in HGFA represents a relatively simple allosteric mechanism involving only one surface loop, which directly links the effector-binding site with the active site. This contrasts with more complex and less well understood allosteric systems, such as the catalytic regulation of the bacterial serine protease DegS by its PDZ domain, where peptide (=effector) binding to the PDZ domain is associated with numerous short- and long-range conformational changes that ultimately form a competent active site [65,66].

Whereas allostery of DegS and other protease systems reflect a physiologically relevant regulation system, the situation for the Fab40–HGFA system is less clear. There is no known effector that binds to the Fab40 epitope region. However, it is quite intriguing that the epitope significantly overlaps with the exosite II region of thrombin [71] and with the effector-binding regions of coagulation Factors IX and X [72,73]. Figures 6(A) and 6(B) illustrate the structural epitope of Fab40 in comparison with thrombin exosite II (modelled on HGFA) located at the 'back side' of the substrate-binding cleft. Of note, the exosites of thrombin, Factor IX and Factor X comprise a cluster of functionally important arginine and lysine residues, which are not present in HGFA. Nevertheless, the close spatial correspondence suggests that the underlying allosteric mechanisms could be related. Exosite binding by effectors, such as an anti-thrombin antibody, heparin and pro-thrombin fragment-2, induces conformational changes at the active-site region of coagulation factors [41,74,75]. Therefore we propose that the HGFA allosteric switch mechanism may present a paradigm for exosite allostery of coagulation factors involving conformational changes of the 99-loop and perhaps also of the 60-loop.

ANTIBODIES THAT INCREASE PROTEASE ACTIVITY

The highly specific allosteric inhibition by Fab40 raises the intriguing possibility that antibodies could be used to achieve the opposite outcome, i.e. the allosteric activation of a protease. Activating antibodies could be beneficial for therapeutic purposes that require increased protease activity. Such an approach could benefit from the highly allosteric nature of many proteases and regulation mechanisms that often involve the structural rearrangement of one or several surface-exposed loops proximal to the active site. It seems possible that an antibody could serve as a surrogate of a natural regulator in ordering of the active-site region and conferring catalytic competency. There are precedents for antibody-mediated protease activation from studies of several trypsin-like serine proteases (Clan PA, family S1). Yoshihara et al. [76] reported a 3-fold increase in catalytic activity of the outer membrane serine protease D2 by a monoclonal antibody. Scheiflinger et al. [77] identified monoclonal antibodies that can serve as a surrogate for cofactor VIII in activating coagulation Factor IXa. Although the effects were relatively modest, it shows that antibodies can mimic some functions of a natural allosteric activator. An anti-Factor IXa antibody identified by the same group enhanced Factor IX activity by increasing the affinity of cofactor binding to Factor IXa [78]. The structural determinants for the antibody-mediated Factor IX activity enhancement have not been elucidated and, therefore, it is presently unknown whether these antibodies act by allosteric mechanisms. The first structural insight into an activity enhancing antibody was obtained recently. Menez et al. [79] solved the structure of antibody (Fab) bound to PSA (prostate-specific antigen, also called kallikrein-3) showing that that Fab binds outside the substrate-binding cleft and contacts several surface loops, including the 170- and the long 99-loop. Although the molecular mechanism by which the antibody enhances PSA activity still remained elusive, the structure seems to implicate two substrate-interacting surface loops as being part of the mechanism. Ultimately, structural studies of activity-enhancing antibody–protease complexes may be used as a screening tool to identify allosteric 'hot spots', which

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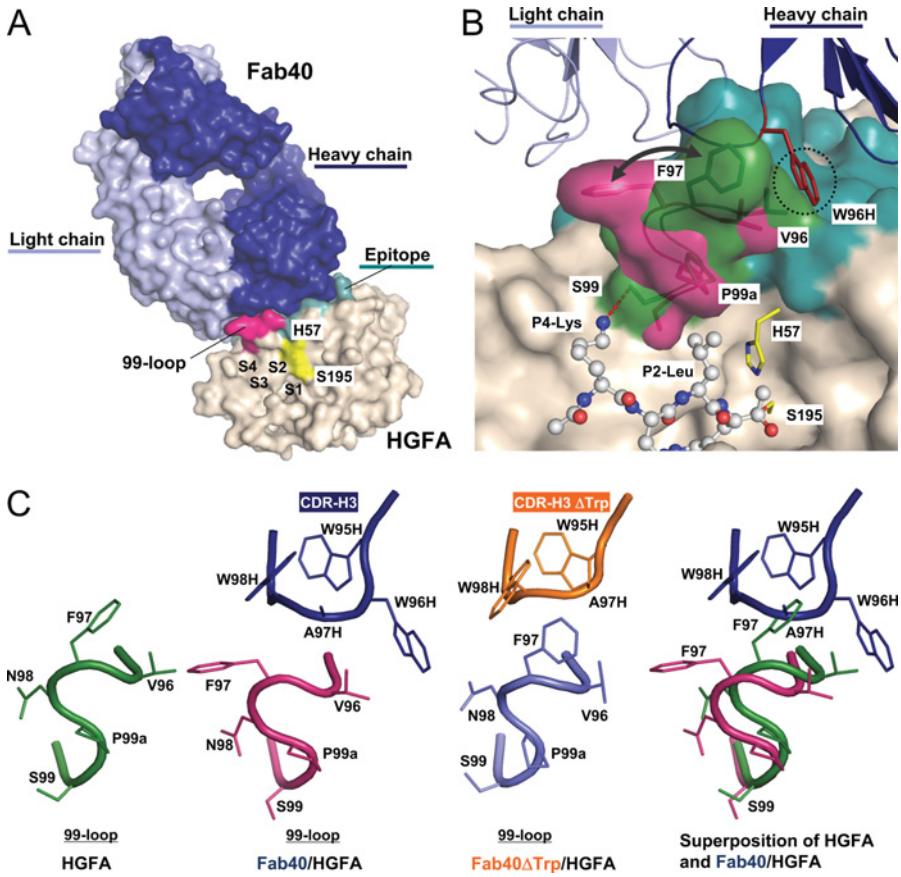

Figure 5 Allosteric inhibition mechanism

(**A**) The Fab40–HGFA complex (PDB code 3K2U) with Fab light and heavy chains in light and dark blue respectively. The 99-loop is coloured in pink, His[57] and Ser[195] of the catalytic triad are in yellow, whereas the Fab40 epitope is coloured in teal. (**B**) A close-up view of the active site of HGFA (beige surface). The peptidic inhibitor KQLR is in ball and stick representation (grey, carbon atoms; blue, nitrogen atoms; red, oxygen atoms). The two different conformations of the 99-loop are coloured in pink (inactive conformation, Fab40–HGFA complex) and green (active conformation, Fab40ΔTrp–HGFA–KQLR peptide complex, PDB code 2WUC). The Trp96H in the CDR-H3 loop of Fab40 is in stick representation coloured red and circled with black dotted lines. (**C**) The different conformational states of the 99-loop of HGFA. The 99-loop in the structure of KD1–HGFA (PDB code 1YC0) (coloured green) is in the active conformation, while a significant change in the conformation is observed in the Fab40-bound state (coloured pink). Deletion of Trp96H (to reduce the length of the CDR-H3 loop) in the structure of Fab40ΔTrp–HGFA (PDB 2WUB) was sufficient to return the 99-loop to the active conformation. Superposition of the 99-loops illustrates the movement of the 99-loop main-chain as well as side-chain residues. Single letter amino acid codes are used in the Figure.

may be amenable to structure-based design of allosterically acting peptidic or small-molecule inhibitors [80–82].

ANTIBODIES AGAINST 'PSEUDO-PROTEASES'

Serine protease homologues or 'pseudo-proteases' are a group of proteins having high structural homology with serine proteases but which lack catalytic activity, mainly owing to detrimental substitutions of catalytic triad residues. Examples of human pseudo-proteases with the chymotrypsin fold include HGF [83,84], MSP [85], Protein Z [86], haptoglobin [87,88], azurocidin [89,90] and polyserase-2 [91]. Recent studies on some of these serine protease homologues demonstrated that the pseudo-protease domain has adapted to interact with protein partners utilizing the pseudo-active site [83,84]. Therefore antibodies may target the pseudo-active-site region much in the same way as observed for HGFA and matriptase, perhaps even providing allosteric regulation of the pseudo-active sites.

An interesting example is HGF, a plasminogen-like growth factor, which is involved in development, morphogenesis and tissue regeneration [92,93]. HGF is the ligand for the receptor tyrosine kinase Met and the HGF/Met system has been implicated in numerous types of cancer. Similar to plasminogen, pro-HGF is activated by proteolytic cleavage at the Arg[494]–Val[495] bond to generate an active disulfide-linked heterodimer, consisting of a Kringle domain-containing α-chain and a pseudo-protease domain (HGF-β) with chymotrypsin fold. In HGF-β the two catalytic residues His[57] and Ser[195] are replaced with glutamine and tyrosine (Figure 7A). The activation cleavage results in the ordering of the pseudo-active site to allow for productive binding to the Met receptor [83,84]. The Met contacts on HGF are at the pseudo-active site (Figure 7B), and to some extent mimic the binding of KD1 to the HGFA active site (Figures 7C and 7D). The close relationship of HGF-β to true proteases extends to the critical role of N-terminal insertion into the 'activation pocket', which in the case of HGF-β stabilizes the conformation of the pseudo-active site for Met binding [94]. Thus the HGF pseudo-active-site region constitutes a promising antibody target region for anti-cancer therapy. Indeed, Burgess et al. [95] identified an anti-HGF antibody that binds to the HGF-β chain and blocks Met binding. The epitope was not described in detail but part of it is in

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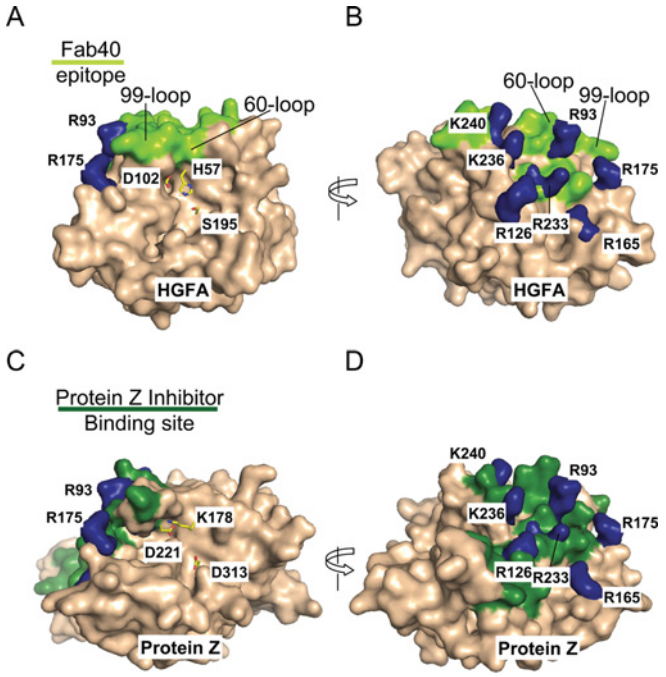

Figure 6 Thrombin exosite II as paradigm

The Fab40 epitope on HGFA (light green) has significant overlap with a region corresponding to exosite-II in thrombin [front view in (**A**) and rear view in (**B**)]. Arginine and lysine residues (blue) in the thrombin exosite-II are critical for effector binding. Protein Z has a pseudo-protease domain with two of the catalytic triad residues (His^{57} and Ser^{195}) mutated to Asp^{313} and Lys^{178}. The Protein Z inhibitor-binding site on Protein Z overlaps with a region corresponding to thrombin exosite-II [front view in (**C**) and rear view in (**D**)]. Single letter amino acid codes are used on the Figure.

proximity to the Met-binding region [96], including the 60-loop and the N-terminus (Figures 7A and 7B). Therefore one aspect of the antibody's inhibition mechanism may be allostery and/or steric hindrance of Met binding to HGF-β. The related pseudo-protease MSP (having the catalytic triad residues His^{57}, Asp^{102} and Ser^{195} replaced with glutamine, glutamine and tyrosine) also uses its trypsin-like β-chain to interact with its cognate receptor RON [85,97] and its regulation and receptor interaction probably follows the HGF paradigm.

Protein Z is a cofactor for a serpin called ZPI (Protein Z-dependent inhibitor), which inactivates coagulation Factor Xa [98]. The crystal structure of the Protein Z–ZPI complex [86] reveals that the Protein Z pseudo-protease domain (having the catalytic triad residues His^{57} and Ser^{195} replaced with aspartate and lysine) utilizes a region corresponding to thrombin exosite II for ZPI interaction (Figures 6C and 6D). Another example is haptoglobin (having the catalytic triad residues His^{57} and Ser^{195} replaced with lysine and alanine), whose function is to capture haemoglobin in plasma followed by clearance of the haptoglobin–haemoglobin complex via binding to CD163 on monocytes [87]. The haptoglobin–CD163 binding is mediated by the pseudo-protease domain of haptoglobin (β-chain), which adopts a chymotrypsin fold [99]. Nielsen et al. [88] determined that the binding of haptoglobin to CD163 is mediated by the 170-loop of the pseudo-protease domain.

An intriguing example of a pseudo-protease with a zinc metalloprotease-like fold is Shh (Sonic hedgehog), a secreted morphogen that promotes cellular differentiation during embryogenesis, but which is also implicated in many types of cancer [100]. The Shh fold is similar to that of bacterial lysostaphin-type peptidases such as LytM and D-Ala–D-Ala metalloproteases such as VanX and D-Ala–D-Ala carboxypeptidase [101–103]. Recent structural insight shows that Shh interacts with its negative regulator Hhip and with the transmembrane receptor Patched through its pseudo-active site groove [104]. An antibody that blocks this interaction, 5E1, was mapped to residues located at the pseudo-active-site groove [104–106]. In agreement, the recent structure of the Fab5E1–Shh complex revealed that the antibody uses all six CDR loops to bind into the pseudo-active-site groove of Shh and that the structural epitope largely overlaps with the binding site of the negative regulator Hhip [107]. Therefore generally speaking the interaction of the 5E1 antibody with Shh is akin to anti-protease antibodies Fab58 and FabE2 in that they all inhibit by targeting the (pseudo-) active-site groove, thereby impeding interaction with substrate–protein partners by a steric hindrance mechanism.

CONCLUSIONS

Recently determined Fab–protease structures provide detailed molecular insight into two fundamentally different mechanisms by which antibodies can reduce the catalytic activity of serine proteases. The antibodies discussed in the present study garnered interest because they affect protease activity. We have learned that they all interact with protruding surface loops on either side of the substrate-binding cleft. These loops serve as a 'grip' for projecting CDR loops into the cleft, or as a movable element to impose allosteric inhibition. The allosteric mechanism of Fab40 takes advantage of the inherent flexibility of surface-exposed loops surrounding the substrate-binding cleft. Such allosteric influences seem likely to be part of the regulation conferred by physiological interactors. It is probable that antibodies can bind other loops and induce allosteric inhibition, e.g. the 60-, 140-, 180- or 220-loops, all of which are critical in shaping the subsites and in interacting with the substrate. The allosteric sites offer the advantage of relatively poor sequence conservation among proteases and thus of relatively high specificity for antibodies directed against them. An added benefit of allosteric inhibitors, such as Fab40, is that they are safeguarded from inadvertent proteolysis, since the epitope is situated outside the catalytic cleft. Furthermore, structural studies of allosteric antibody–protease complexes may identify allosteric 'hot spots' for small-molecule inhibitor design. For instance, in the case of Fab40–HGFA complex, the interaction of Trp96H with a large hydrophobic pocket ('hot spot') is critical in stabilizing the non-competent 99-loop conformation, yet the existence of this pocket could not have been predicted from other HGFA structures. These allosteric 'hot spots' may be amenable to structure-based design of allosterically acting peptidic or small molecules that may act as inhibitors or as activators [80].

Most physiological and pathological processes seem to involve co-operation of many proteases, which form proteolytic 'cascades' or local proteolytic networks [58,108,109]. For specific therapeutic purposes, it may thus be of advantage to simultaneously target more than one protease but without sacrificing specificity. Recent breakthroughs in antibody engineering indicate that it may be possible to neutralize more than one protease with a single antibody. First, a bispecific antibody targeting two different antigens (one by each Fab arm), was recently approved as a therapeutic (Removab®). Secondly, an astounding development was the generation of the DAFs (dual-action Fabs), where one Fab binds to two different antigens [110]. There is great hope that the exciting progress in antibody engineering and progress in identifying

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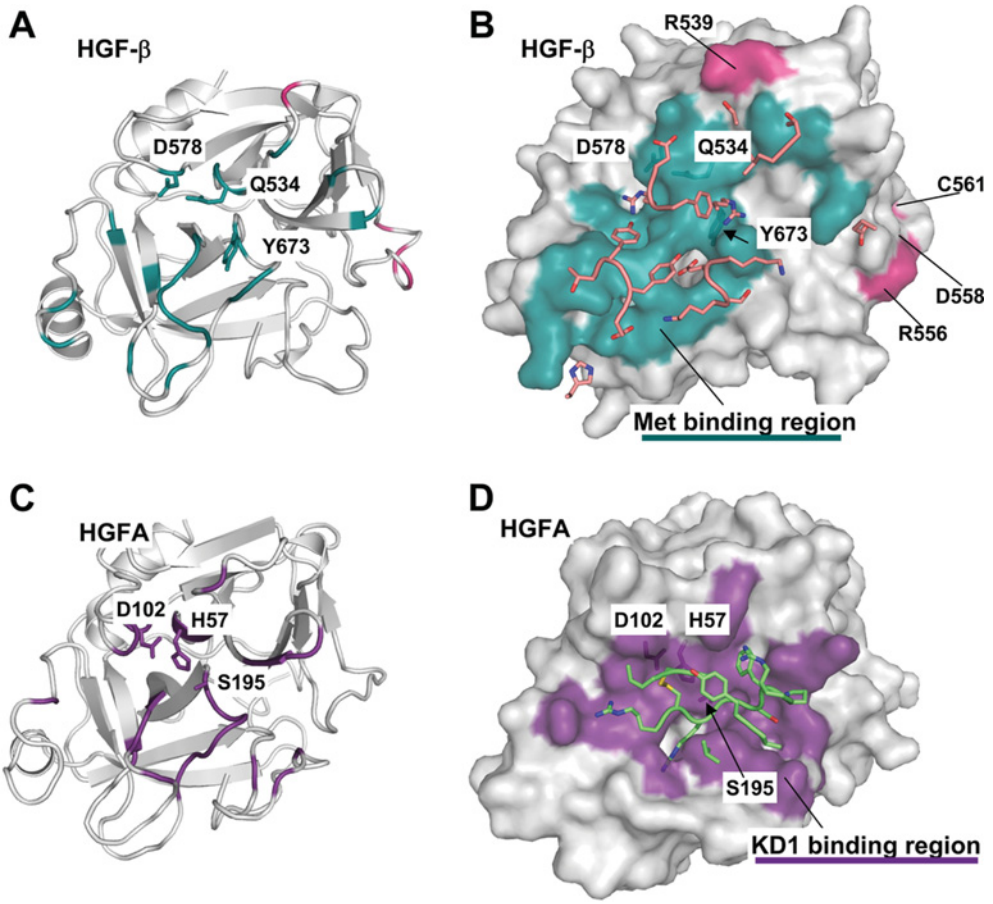

Figure 7 Functional significance of pseudo-active sites

(**A**) The pseudo-protease HGF-β from the crystal structure of the receptor tyrosine kinase Met in complex with the HGF-β (PDB code 1SHY), which adopts a trypsin-like fold. Two of the 'catalytic triad' residues in HGF-β are mutated (Gln^{534} instead of His^{57} and Tyr^{673} instead of Ser^{195}). (**B**) Residues of Met interacting with HGF-β (grey surface) are salmon coloured in cartoon style. The Met-binding region (4 Å cut-off, teal coloured) significantly overlaps with the binding region of a canonical Kunitz domain inhibitor (KD1) of HGFA (see **D**). Part of the epitope for an anti-HGF antibody is located in proximity of the Met-binding region [96] (residues Arg^{539}, Arg^{556}, Asp^{558} and Cys^{561}, coloured pink). (**C**) The protease domain of HGFA from the crystal structure of HGFA in complex with KD1 (PDB 1YC0). The KD1-binding region is coloured in purple (catalytic triad residues: His^{57}, Asp^{102} and Ser^{195} in stick representation). (**D**) Residues of HGFA (grey surface) interacting with KD1 are coloured purple (4 Å cut-off) and the KD1 contact residues (green) are shown in cartoon style. Single letter amino acid codes are used in the Figure.

disease-promoting proteases will ultimately result in the development of therapeutic anti-protease antibodies.

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