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Cystatins as Calpain Inhibitors: Engineered Chicken Cystatin- and Stefin B-Kininogen Domain 2 Hybrids Support a Cystatin-Like Mode of Interaction with the Catalytic Subunit of μ-Calpain

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Within the cystatin superfamily, only kininogen domain 2 (KD2) is able to inhibit μ- and m-calpain. In an attempt to elucidate the structural requirements of cystatins for calpain inhibition, we constructed recombinant hybrids of human stefin B (an intracellular family 1 cystatin) with KD2 and ΔL110 deletion mutants of chicken cystatin-KD2 hybrids. Substitution of the N-terminal contact region of stefinB by the corresponding KD2 sequence resulted in a calpain inhibitor of K_i = 188 nM. Deletion of L110, which forms a β-bulge in family 1 and 2 cystatins but is lacking in KD2, improved inhibition of μ-calpain 4- to 8-fold. All engineered cystatins were temporary inhibitors of calpain due to slow substrate-like cleavage of a single peptide bond corresponding to Gly9-Ala10 in chicken cystatin. Biomolecular interaction analysis revealed that, unlike calpastatin, the cystatin-type inhibitors do not bind to the calmodulin-like domain of the small subunit of calpain, and their interaction with the μ-calpain heterodimer is completely prevented by a synthetic peptide comprising subdomain B of calpastatin domain 1. Based on these results we propose that (i) cystatin-type calpain inhibitors interact with the active site of the catalytic domain of calpain in a similar cystatin-like mode as with papain and (ii) the potential for calpain inhibition is due to specific subsites within the papain-binding regions of the general cystatin fold.

Key words: Calpastatin / Kininogen / Papain / Stefin B / Surface plasmon resonance / Temporary inhibition.

Introduction

The two ubiquitous calpains, μ-calpain and m-calpain, are intracellular, Ca^{2+}-dependent cysteine proteinases that have been implicated in many important cellular functions and various pathologies (see Sorimachi *et al*., 1997; Carafoli and Molinari, 1998; Suzuki and Sorimachi, 1998 for recent reviews). They consist of distinct (yet homologous) large (L-)subunits (80 kDa) und a common small (S-)subunit (30 kDa). On the basis of sequence comparisons, the L-subunit has been predicted to contain four and the S-subunit two domains. Whereas the catalytic domain (domain II) of the L-subunit shows a weak sequence homology to papain, both the L-subunit and the S-subunit contain a Ca^{2+}-binding calmodulin-like domain (CaMLD). Until recently, only three-dimensional structures of the L-CaMLD were known (Blanchard *et al*., 1997; Lin *et al*., 1997), and hypotheses on the molecular mechanisms of activation and inhibition of calpains have been contradictory. Meanwhile, two groups have published crystal structures of the Ca^{2+}-free, inactive form of m-calpain, revealing the molecular architecture of this multidomain protein (Hosfield *et al*., 1999; Strobl *et al*., 2000). In these structures, the catalytic domain (II) appears disrupted into two subdomains (IIa and IIb), explaining the inactivity of calpain in the absence of calcium. Activation should involve a 'fusion' of the two subdomains, leading to a functional papain-like catalytic domain (Hosfield *et al*., 1999; Strobl *et al*., 2000). As long as the structure of a Ca^{2+}-activated calpain is not known, a number of questions concerning the molecular mechanisms of interaction with substrates and inhibitors remain open.

Ca^{2+}-activated μ- and m-calpain are controlled by a very specific intracellular protein inhibitor, calpastatin. Calpastatin contains four repeats of the inhibitory unit, each of which can inhibit calpain independently, but is not able to inhibit other cysteine proteinases of the papain superfamily (Maki *et al*., 1987; Emori *et al*., 1988).

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Conversely, calpain is not inhibited by most cystatins (Crawford, 1987), the only exceptions being the kininogens. The second cystatin-like kininogen domain (KD2) was shown to be responsible for calpain inhibition (Salvesen *et al.*, 1986). Whereas the interaction of calpain with calpastatin has been studied in some detail (Takano *et al.*, 1995), the interaction between calpains and kininogens remains to be investigated.

No relation can be found between cystatins and calpastatin on the basis of sequence homology. They possess a well defined three-dimensional structure (Turk and Bode, 1991), in contrast to the proposed disordered structure of calpastatin domains (Uemori *et al*., 1990). While attempting to identify the structural requirements for calpain inhibition by cystatins, we have shown earlier that substitution of the three chicken cystatin contact regions (N-terminal trunk, first and second hairpin loop) by the corresponding KD2 sequences in a single and combined manner resulted in up to 1000-fold improved calpain inhibition (Auerswald *et al*., 1996). However, inhibition of m-calpain by these chimeric proteins was found to be temporary. In the present work, we investigate the mechanism of temporary inhibition of μ-calpain by chicken cystatin-KD2 hybrids and the potential reasons for it. The second disulphide loop (Cys95-Cys115) of chicken cystatin is extended by one leucine residue (L110) as compared to the corresponding sequence in KD2 (see Figure 1); L110 forms a β-bulge of the terminal β-sheet that is conserved in family 1 and 2 cystatins (Bode *et al.*, 1988; Stubbs *et al*., 1990). This difference could be responsible for non-optimal binding of the chicken cystatin-KD2 hybrids to calpain resulting in temporary inhibition. In order to test our hypothesis we constructed chicken cystatin-KD2 ΔL110 deletion mutants and compared them with the corresponding full-length chicken cystatin-KD2 hybrids.

Furthermore, we attempt to clarify whether calpain inhibition by engineered cystatins requires a family 2 (chicken cystatin) scaffold or is rather due to the presence of calpain-binding sequences (as in KD2) on any cystatin fold. For this purpose we constructed cystatin-KD2 hybrids using as parent molecule a member of family 1 of the cystatin superfamily, human stefin B. Like all family 1 members, stefin B conserves the general architecture of cystatins but, in contrast to chicken cystatin or KD2, occurs intracellularly and lacks disulphide bridges. Stefin B has a shorter N-terminal 'trunk' and a wider second hairpin loop than chicken cystatin (Stubbs *et al*., 1990).

The interaction of the chimeric inhibitors with human μ-calpain and with a recombinant fragment of its small subunit was studied by real time biomolecular interaction analysis using surface plasmon resonance technology. A hypothesis is presented on the mode of interaction of calpain with cystatin-type protein inhibitors and is compared with the present knowledge on its interaction with calpastatin.

Results

Gene Design for the Chimeric Proteins

Figure 1 shows the amino acid sequences of the engineered proteins compared with those of the parent proteins. The ΔLeu110 chicken cystatin-KD2 hybrids were prepared from the corresponding chicken cystatin-KD2

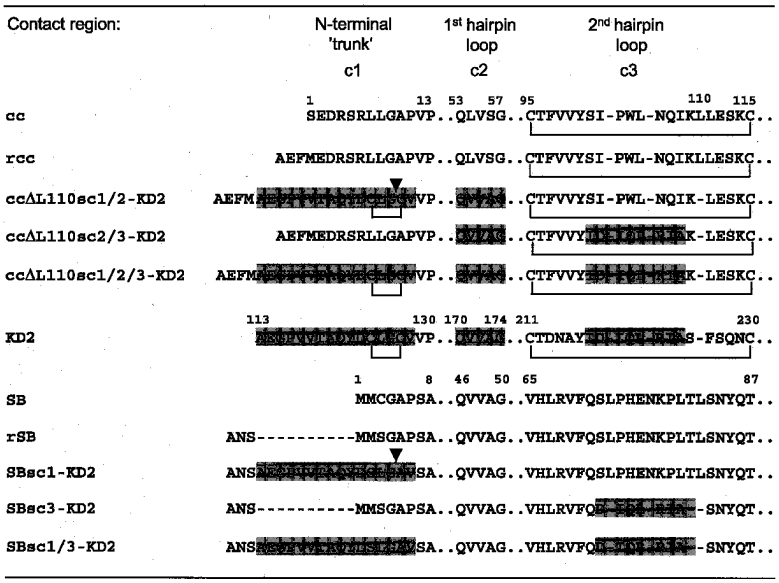

Fig. 1 Scheme of the Chicken Cystatin- and Stefin B-Kininogen Domain 2 Hybrids.
The contact regions of stefin B and chicken cystatin with papain (c1, c2, c3) were substituted by the corresponding sequences of human kininogen domain 2 (grey) in a single and combined manner (hybrids sc1, sc3, sc1/2, sc1/3, sc1/2/3). cc, natural chicken cystatin; rcc, recombinant chicken cystatin (AEF-[M1, I29, L89] chicken cystatin); KD2, kininogen domain 2; SB, natural stefin B; rSB, recombinant stefin B; ΔL110, Leu110 deletion mutant of cc. The sequences of chicken cystatin and stefin B were aligned according to topological equivalence in tertiary structure (Stubbs *et al.*, 1990). The peptide bonds cleaved during temporary inhibition of μ-calpain are marked by arrowheads.

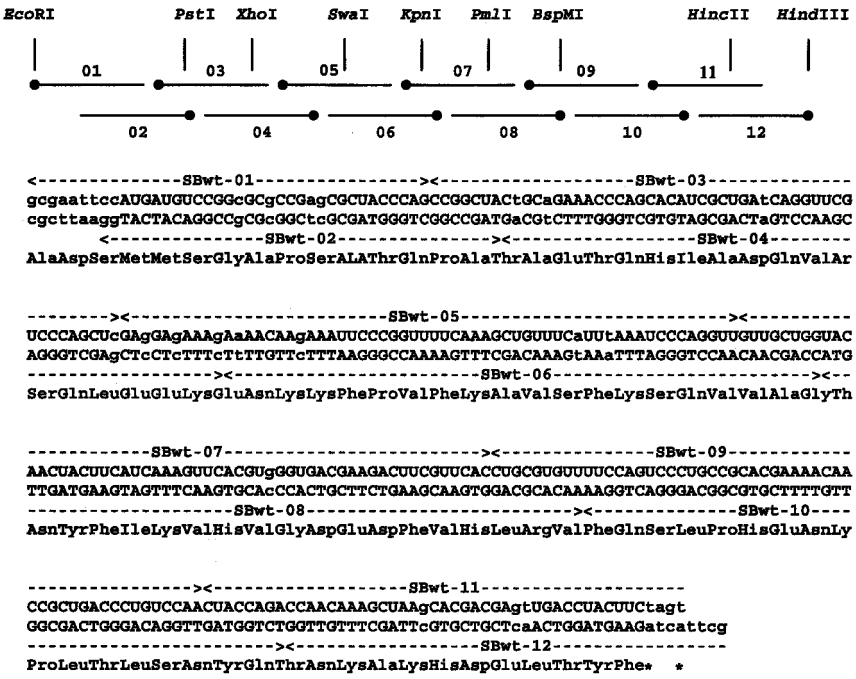

Fig. 2 Synthetic Gene Encoding Human Stefin B.
The upper panel shows the construction of the synthetic gene from 12 individual oligonucleotides (numbered lines) and the distribution of unique restriction sites. The complete DNA and amino acid sequences of ANS-[C3S]-r-stefin B are presented in the lower panel.

hybrids described earlier (Auerswald *et al*., 1996) by PCR mutagenesis and ligated into the expression vector pIN-III-ompA2 to allow periplasmic expression in *E. coli*. The stefin B-KD2 hybrids were constructed following the same general strategy as described previously for the chicken cystatin-KD2 hybrids (Auerswald *et al*., 1996). Three stefin B-KD2 hybrids were designed, carrying the putative contact regions c1 (N-terminus) and c3 (second hairpin loop) of KD2 in a single and combined manner. Since contact region c2 (first hairpin loop) of stefin B and KD2 are identical, further substitutions were not necessary. A synthetic gene for stefinB (Figure 2) was designed and assembled from twelve oligonucleotides according to the method of Khorana (1979). The single cysteine residue in stefin B (Cys3) was substituted by serine in order to avoid dimerisation *via* disulphide bridges. The hybrids were then constructed either by total gene synthesis or by cassette mutagenesis (see Materials and Methods for details). The insertion of an *Eco*RI restriction site at the 5' end of the genes for ligation into the cloning and expression vectors (pUC 18 and pASK 40) involved the introduction of the tripeptide sequence Ala-Asn-Ser- (ANS-) at the N-terminus of the recombinant proteins. Cys124 and Cys127 of the N-terminal KD2 sequence were replaced by Ser and Ala, respectively. Subcloning of the genes into the expression vector pASK 40 enabled periplasmic expression.

Protein Expression, Purification and Characterisation

The ΔLeu110 chicken cystatin-KD2 hybrids were isolated from the periplasmic fractions of the induced *E. coli* cultures and purified to 90–95% homogeneity by affinity chromatography on a carboxymethyl-papain Sepharose column followed by anionic exchange chromatography on a Mono Q column (Figure 3). The identity of the chimeric proteins was verified by N-terminal amino acid sequencing and mass spectrometry. Table 1 summarises the results of expression, purification and characterisation of the hybrids.

The stefin B-KD2 hybrid SBsc1-KD2 (see Figure 1 for explanation of abbreviations) was detected in mg amounts (estimated by Western blotting and papain in-

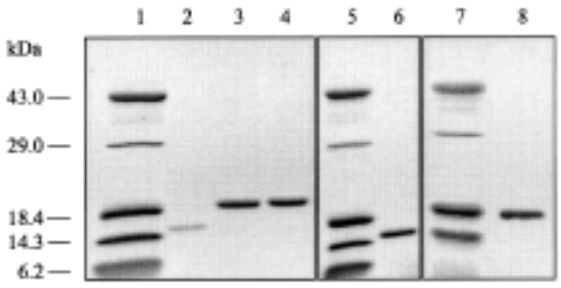

Fig. 3 SDS-PAGE of the Purified Hybrids.
Lanes 1,5,7 show low molecular mass standards. Three to five μg of purified inhibitors were applied: lane 2, ΔL110cc; lane 3, ΔL110ccsc1/2-KD2; lane 4, ΔL110ccsc1/2/3-KD2; lane 6, ΔL110ccsc2/3-KD2 and lane 8, SBsc1-KD2.

Table 1 Expression, Purification and Protein Chemical Characterisation of the ΔL110 Chicken Cystatin-KD2 and Stefin B-KD2 Hybrids.

Hybrid[a]	Expression [mg/l][b]	Purification [mg/l][b]	N-terminal sequence		Molecular mass [Da]
ΔL110cc	3.5	1.6	AEFME	>95%	13386.1
ΔL110ccsc1/2-KD2	3.2	1.6	AEF	>95%	13865.9
ΔL110ccsc2/3-KD2	0.8	0.3	AEF	>95%	13327.0
ΔL110ccsc1/2/3-KD2	2.0	0.8	AEFMAEG	>90%	13838.7
SBsc1-KD2	5.5	1.5	ANSAEGPV	>95%	12379.7

[a]See Figures 1 and 6 for abbreviations.
[b]mg of active inhibitor per litre of *E. coli* cell culture.

Table 2 Equilibrium Dissociation Constants (K_i) and Rate Constants (k_{on}, k_{off}) of the Complexes of the Inhibitors with Different Cysteine Proteinases.

Inhibitor[a]	Papain			Cathepsin L			μ-Calpain
	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i (nM)	K_i (nM)
r-chicken cystatin	1.3×10^{7}[b]	1.8×10^{-5}[b]	0.0014[b]	7.8×10^{7}[b]	6.7×10^{-5}[b]	0.0009[b]	75000
ΔL110cc	2.8×10^{7}	1.3×10^{-4}	0.0046	5.5×10^{7}	3.5×10^{-4}	0.0064	920*
ccsc1/2-KD2	3.2×10^{7}[b]	4.1×10^{-5}[b]	0.0013[b]	6.3×10^{7}[b]	3.3×10^{-4}[b]	0.0052[b]	127*
ΔL110ccsc1/2-KD2	2.9×10^{7}	9.7×10^{-5}	0.0034	2.5×10^{7}	3.7×10^{-4}	0.0148	29*
ccsc2/3-KD2	1.9×10^{7}[b]	6.3×10^{-5}[b]	0.0033[b]	4.0×10^{7}[b]	1.6×10^{-4}[b]	0.0040[b]	≥30000
ΔL110ccsc2/3-KD2	4.5×10^{6}	1.1×10^{-4}	0.0244	1.3×10^{7}	2.8×10^{-4}	0.0215	≥ 5000
ccsc1/2/3-KD2	5.8×10^{6}[b]	2.8×10^{-4}[b]	0.0480[b]	3.9×10^{6}[b]	1.7×10^{-3}[b]	0.436[b, *]	380*
ΔL110ccsc1/2/3-KD2	2.9×10^{6}	2.0×10^{-4}	0.0690	2.8×10^{6}	1.2×10^{-3}	0.428*	100*
$KD2_{prot}$	7.3×10^{6}[b]	1.2×10^{-4}[b]	0.0160[b]	2.2×10^{7}[b]	3.3×10^{-4}[b]	0.0150[b]	5.4
L-kininogen	2.0×10^{7}[b]	6.4×10^{-5}[b]	0.0032[b]	3.0×10^{7}[b]	1.1×10^{-4}[b]	0.0035[b]	1.0
SBsc1-KD2	6.6×10^{6}	7.6×10^{-5}	0.0120	4.0×10^{6}	4.1×10^{-5}	0.0104	188*
r-stefin B	8.9×10^{6}	5.7×10^{-5}	0.0064	2.1×10^{7}	1.8×10^{-4}	0.0086	≥60000

[a] See Figures 1 and 6 for abbreviations.
[b] Data taken from Auerswald *et al.* (1996).
* Temporary inhibition.

hibitory activity) in the periplasm, whereas SBsc3-KD2 and SBsc1/3-KD2 formed inclusion bodies. The hybrid SBsc1-KD2 was purified to 95% homogeneity and characterised by SDS-PAGE (see Figure 3), reversed-phase HPLC (data not shown), N-terminal amino acid sequencing and mass spectrometry (Table 1).

Inhibitory Profiles of the Hybrids

The equilibrium dissociation constants (K_i) as well as the association and dissociation rate constants (k_{on}, k_{off}) of the complexes formed between the inhibitors and different cysteine proteinases are summarised in Table 2. Inhibition of papain and cathepsin L by the chicken cystatin hybrids was marginally less than that of the parent molecules; nevertheless, all hybrids remained tight-binding inhibitors of these enzymes (K_i in the pM range), suggesting that neither deletion of the Leu110 residue in the chicken cystatin hybrids nor the N-terminal substitution of stefin B with a KD2 sequence had essentially altered the general cystatin fold required for tight-binding interactions. On the other hand, deletion of Leu110 in the chicken cystatin hybrids resulted in a 4-5-fold improvement of calpain inhibition. While the K_i of recombinant stefin B for the inhibition of μ-calpain is at least 60 μM, substitution of its N-terminus by that of KD2 in the SBsc1-KD2 hybrid resulted in 300-fold improved calpain inhibition (K_i=188 nM).

Temporary Inhibition of μ-Calpain

Inhibition of μ-calpain by the ΔL110 chicken cystatin-KD2 and stefin B-KD2 hybrids was shown to be temporary. After rapid initial inhibition, the activity of calpain reappeared and increased slowly until a second stable equilibrium was reached. Figure 4 shows a typical inhibition experiment of μ-calpain by ΔL110ccsc1/2-KD2.

The time course of the inhibition experiment can be explained by a conversion of the inhibitor into a 'modified' form of much lower affinity. This putative conversion was confirmed by SDS-PAGE and N-terminal amino acid sequencing (Figure 5). After incubation of the hybrids ccΔL110sc1/2-KD2 and SBsc1-KD2 with μ-calpain, a second band was detected migrating at a lower apparent

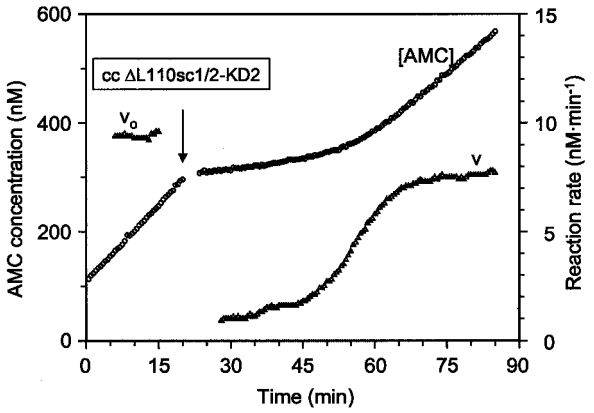

Fig. 4 Temporary Inhibition of μ-Calpain by ΔL110ccsc1/2-KD2.
Typical inhibition experiment showing the time course of concentration of the fluorescent reaction product (AMC, circles) and the reaction rate (triangles) calculated within a moving window of 10 min (v). After a constant reaction rate (v_0) had been obtained with μ-calpain (10 nM), ΔL110ccsc1/2-KD2 (280 nM) was added (arrow).

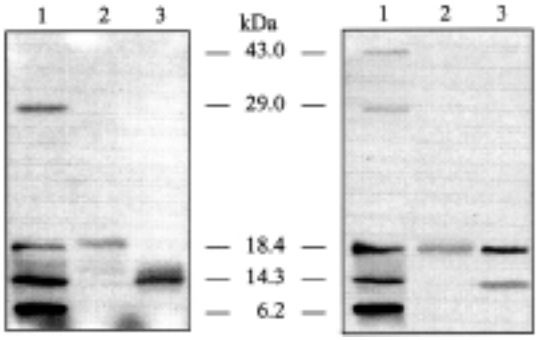

Fig. 5 Analysis of Temporary Inhibition by SDS-PAGE.
ΔL110ccsc1/2-KD2 (left) and SBsc1-KD2 (right) were incubated with μ-calpain for 90 min. Samples taken at time 0 (lane 2) and 90 min (lane 3) were analysed by SDS PAGE and N-terminal sequencing. The new sequences found were CVVP… for ΔL110ccsc1/2-KD2 and AVSA… for SBsc1-KD2, resulting from cleavage behind Gly18 or Gly17, respectively (see Figure 1 for the complete sequences). Lane 1, low molecular mass standards.

molecular mass. N-terminal sequencing of the incubated mixtures revealed that the conversion is due to cleavage of a single peptide bond, Gly18-Cys19 in the chicken cystatin-KD2 hybrid, and Gly17-Ala18 in the stefinB-KD2 hybrid. These bonds correspond to Gly9-Ala10 in natural chicken cystatin and Gly4-Ala5 in natural stefin B (see Figure 1) involving the Gly residue that is highly conserved within the cystatin superfamily (Turk and Bode, 1991).

Biomolecular Interaction Analysis

The chicken cystatin-KD2 hybrid ccΔL110sc1/2-KD2and the stefin B-KD2 hybrid SBsc1-KD2 were chosen to investigate the binding of μ-calpain to these cystatin-like inhibitors and to compare it with the binding of μ-calpain to recombinant inhibitory calpastatin domain 1 (rCD1; Emori *et al*., 1988). For real-time biomolecular interaction analysis the inhibitors were immobilised on CM5 chips *via* amino groups. The interaction of μ-calpain with the immobilised inhibitors was analysed in the presence and in the absence of calcium. In the presence of 0.2 mM $CaCl_2$ we observed a strong binding of μ-calpain to rCD1; but also $KD2_{prot}$ (KD2 obtained by limited proteolysis of L-kininogen) as well as the ΔL110chicken cystatin-KD2 and stefin B-KD2 hybrids were able to bind the injected μ-calpain (Figure 6A). In contrast, Ca^{2+}-activated calpain did not bind to immobilised chicken cystatin or stefin B, which were used as negative controls. When μ-calpain was injected in the absence of calcium, no binding to the immobilised inhibitors was observed (data not shown).

Calpastatin peptide B (CPB), a synthetic peptide of 27 amino acids comprising the minimal inhibiting unit of calpastatin domain 1, has been shown to be a competitive inhibitor and is thus supposed to bind to the active site of calpain (Maki *et al*., 1988; Kawasaki *et al*., 1989; Crawford *et al*., 1993). Therefore we performed competition experiments with this peptide. For complex formation, CPB was added to Ca^{2+}-activated μ-calpain (0.2 mM Ca^{2+} for 5 min) in a molar ratio of 20:1. When the mixture was passed over the chips, only immobilised rCD1 was able to bind μ-calpain, whereas the immobilised chicken cystatin-KD2 and stefin B-KD2 hybrids as well as $KD2_{prot}$ did not display any binding (Figure 6 B). Similarly, binding of μ-calpain to the cystatin-like inhibitors was completely abolished when the activated enzyme was pre-incubated with an excess of whole rCD1 (data not shown).

It has been shown previously that the inhibitory domain 1 of calpastatin is able to bind the calmodulin-like domains of the large subunit (domain IV) and of the small subunit (domain VI) of calpain (Takano *et al*., 1995). Therefore we investigated the interaction of KD2 as well as of cystatin- and stefin B-KD2 hybrids with a recombinant 21 kDa fragment containing the complete CaMLD (domain VI) of the small subunit (Figure 6C). Clearly, neither immobilised KD2 nor the immobilised chicken cystatin-KD2 and stefin B-KD2 hybrids were able to bind the 21kDa protein. In contrast, immobilised rCD1 exhibited a fast, strong interaction with this calpain fragment. Immediately after the described binding experiments, the immobilised inhibitors were able to bind again activated calpain and carboxymethylated papain (data not shown), proving their integrity and the validity of the results obtained.

Discussion

Due to the lack of structural information on Ca^{2+}-activated calpain and KD2, the precise molecular mechanism of their interaction is still unknown. However, from a sequence alignment of KD2 with other members of the cystatin superfamily and from its inhibitory profile (Auerswald

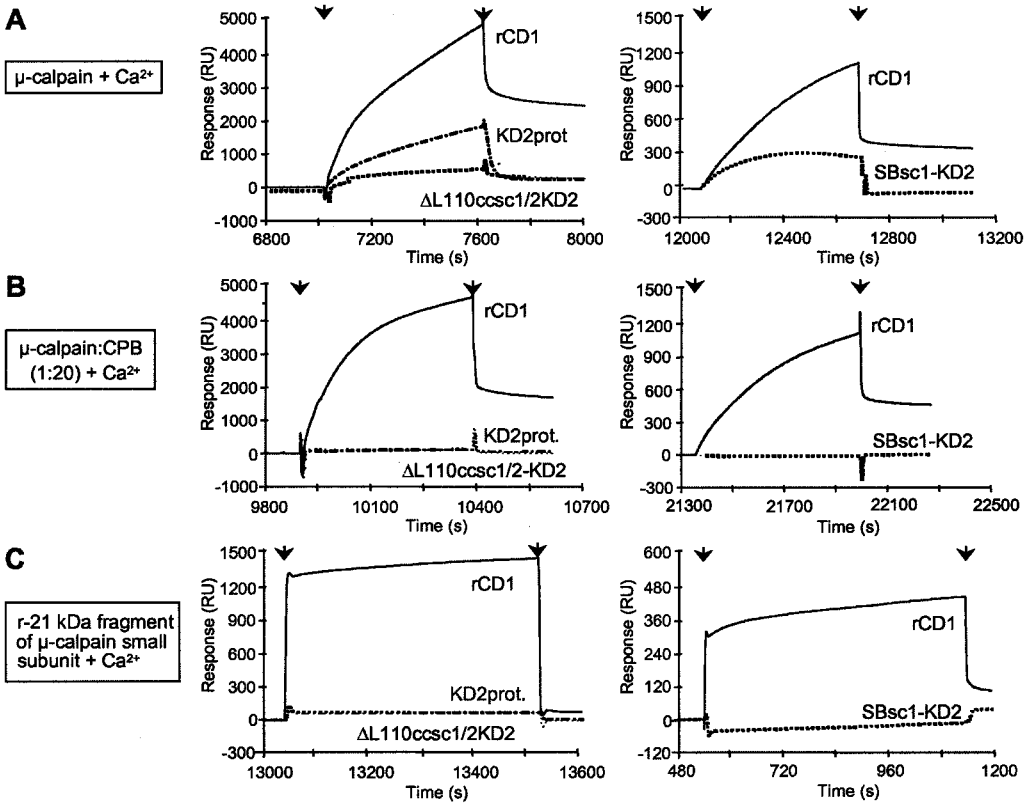

Fig. 6 Binding of μ-Calpain to Immobilised Cystatin- und Calpastatin-Type Inhibitors.
Overlay plots of typical BIAcore sensorgrams showing the interaction of the immobilised inhibitors with injected μ-calpain (A, B) and a recombinant fragment (r-21 kDa) of its small subunit (C), respectively, in the presence of calcium ions. A preincubated mixture of Ca^{2+}-activated μ-calpain and calpastatin peptide B (CPB) was injected in competition experiments (B). The panels on the left side show sensorgrams obtained with a chip loaded with recombinant calpastatin domain 1 (rCD1, —), kininogen domain 2 obtained by limited proteolysis ($KD2_{prot}$, –·–), and ΔL110ccsc1/2-KD2 (·····). On the right side, sensorgrams of a second chip loaded with rCD1 (—) and SBsc1-KD2 (·····) are displayed. Arrowheads indicate start (left) and end (right) of injections. The 'bulk' effect of buffer constituents was minimised by subtracting blank sensorgrams (not shown) obtained with immobilised chicken cystatin (left panels) or recombinant stefin B (right panels).

et al., 1996) we expect kininogen domain 2 to have a cystatin-like structure and thus to inhibit calpain by a similar molecular mechanism as it inhibits papain-like cysteine proteinases. Moreover, structural alignment of the catalytic domain (subdomains IIa and IIb) of m-calpain with papain suggested that the functional active site formed after calcium-triggered conformational changes should be similar to the active site of papain (Hosfield *et al.*, 1999; Strobl *et al.*, 2000).

Exchange of the papain-binding regions of chicken cystatin by the corresponding sequences of KD2 rendered chicken cystatin-KD2 hybrids that inhibited m-calpain with equilibrium dissociation constants (K_i) in the higher nM range (Auerswald *et al.*, 1996). In contrast to earlier results (Bradford *et al.*, 1993), a contribution of all three binding regions of the cystatins to calpain inhibition could be demonstrated (Auerswald *et al.*, 1996), leading us to the hypothesis that the interaction between KD2 and calpain closely resembles the interaction of cystatins with papain-like cysteine proteinases. Our results obtained with the stefin B-KD2 hybrid SBsc1-KD2 support this hypothesis. Stefin B belongs to family 1 of the cystatin superfamily (Turk and Bode, 1991) and differs from chicken cystatin (family 2) and KD2 (family 3) by its intracellular occurrence and lack of disulphide bridges. Its binding mode to cysteine proteinases is well documented by the crystal structure of a stefinB complex with carboxymethylated papain (Stubbs *et al.*, 1990). In the present work, substitution of the N-terminal segment of stefin B by the corresponding sequence of KD2 resulted in a μ-calpain inhibitor with a K_i of 188 nM, suggesting that calpain inhibition is not dependent on the individual scaffold of kininogen domain 2 or chicken cystatin, but is rather due to the presence of calpain-binding sites which may be located within the contact regions of either a family 1, family 2 or family 3 cystatin-fold. As the two Cys residues of the N-terminal segment of KD2 were replaced by Ser and Ala in the SBsc1-KD2 hybrid, the N-terminal 'small' disulphide loop of KD2 seems to be no absolute requirement for calpain inhibition. The corresponding ΔL110ccsc1/2-KD2 hybrid containing these two cysteines is an only 6-fold more potent calpain inhibitor.

Examination of the inhibition profiles of the hybrids against papain and cathepsin L (Table 2) reveals that the

chimeric inhibitors inhibit these enzymes almost as efficiently as the parent molecules. This allows us to assume that the mutations did not significantly affect the general cystatin-like mode of interaction with papain-like cysteine proteinases, although they introduced the additional capability for calpain inhibition. The precise nature of the sequences required for calpain inhibition cannot be deduced from the limited number of hybrid proteins studied in this work, but the N-terminal contact region seems to play a predominant role. New reports on cystatin C and D have indeed shown that the N-terminal regions of cystatins are the most important determinants of inhibitory selectivity for individual papain-like proteinases (Mason *et al*., 1998). On the other hand, changes within the cystatin framework have been found to modulate inhibitory specificity and potency (Gerhartz *et al*., 1997; Hall *et al*., 1998). The latter observations are in accordance with our findings that deletion of the single Leu110 residue in the chicken cystatin-KD2 hybrids improved calpain inhibition 4 to 8-fold (see Table 2). Surprisingly, the very weak but significant μ-calpain inhibition by wild-type chicken (K_i = 75 μM) increased even 80-fold after deletion of L110. Leu110 is not part of a contact region, but its deletion was expected to amend the misannealing of the putative c3 loop of KD2 to the chicken cystatin scaffold (Auerswald *et al*., 1996).

In contrast to the stable inhibition of calpain by KD2, it was found previously that all calpain-inhibiting chicken cystatin-KD2 hybrids were temporary inhibitors (Auerswald *et al*., 1996). In the present work we were able to show that this temporary inhibition is due to selective cleavage of a single peptide bond after the conserved Gly residue within the proposed N-terminal binding regions of the chimeric inhibitors (corresponding to Gly9 in chicken cystatin and Gly4 in stefin B), resulting in N-terminally truncated inhibitors of much lower affinity. We have observed cleavage of the Gly9-Ala10 peptide bond by papain in previous work with deletion mutants of the first and second hairpin loop of chicken cystatin and explained it by substrate-like cleavage of the mutated inhibitors due to distorted binding in one of the two hairpin loop regions required for the formation of a stable complex (Auerswald *et al*., 1995; Machleidt *et al*., 1995). The fact that the recombinant chimeric calpain inhibitors, but not inhibitorily active KD2 (obtained by limited proteolysis of L-kininogen), are cleaved by calpain at the corresponding peptide bond allows three conclusions: (i) the cystatin-type calpain inhibitors interact with the active site of the catalytic subunit of calpain; (ii) their mode of interaction with calpain is similar to that of cystatins with papain-like cysteine proteinases; (iii) in contrast to natural KD2, the chimeric inhibitors fit imperfectly to the active site of calpain and are therefore slowly cleaved in a substrate-like manner. Attempts to generate permanent calpain inhibitors by modification of the cleavage site do not seem promising because mutations in the corresponding region of cystatin C have been shown to decrease its binding affinity for various cysteine proteinases (Björk *et al*., 1995).

Our initial hypothesis that the misalignment of the second disulphide loop of chicken cystatin with the corresponding part of KD2 might be responsible for the temporary calpain inhibition of the chicken cystatin-KD2 hybrids was not confirmed. Although calpain inhibition by all cc-KD2 ΔL110 deletion mutants was slightly improved (see above), they remained temporary inhibitors. Like the chicken cystatin-KD2 hybrids, the stefin B-KD2 hybrid SBsc1-KD2 is also a temporary inhibitor of μ-calpain. Sequence alignment of contact region c3 of stefin B with the corresponding KD2 region displays even greater differences than in the case of chicken cystatin. This may explain why the hybrids SBsc3-KD2 and SBsc1/3-KD2 were not expressed as soluble proteins in *E. coli* periplasmic fractions, but appeared as inclusion bodies in the cytoplasm. The second hairpin loop region appears to play an important role in the maintenance of the overall three-dimensional structure.

For the first time, the binding of whole heterodimeric μ-calpain to kininogen domain 2 and to calpain-inhibiting recombinant cystatin variants was investigated by real time biomolecular interaction analysis and compared with the binding of μ-calpain to the inhibitory domain 1 of calpastatin. The most stringently conserved residues among the four repetitive calpastatin domains are not randomly distributed but cluster in three subdomains designated A, B and C within each domain (Maki *et al*., 1989). Structure-function analysis has revealed that region B is essential for inhibition and that a 27-residue synthetic oligopeptide (calpastatin peptide B, CPB) comprising this region retains inhibitory activity in the low nanomolar range. In this work, we were able to show by real-time biomolecular interaction analysis that binding of μ-calpain to kininogen domain 2 as well as to chicken cystatin-KD2 and stefin B-KD2 hybrids can be prevented by an excess of CPB. Competition of CPB with calpain substrates has been demonstrated previously by kinetic analysis using calpastatin fragments containing the region B (Maki *et al*., 1988; Kawasaki *et al*., 1989). Binding of peptide B near the catalytic centre of calpain has further been supported by competition experiments using small inhibitors (Kawasaki *et al*., 1989; Crawford *et al*., 1993). Based on these earlier reports, our results strongly support the hypothesis that the cystatin-like inhibitors bind to the catalytic domain of calpain.

Conversely, binding of μ-calpain to recombinant calpastatin domain 1 could not be prevented by CPB, indicating that additional binding sites are involved. These results agree with previously published BIAcore experiments demonstrating that non-inhibitory peptides derived from regions A and C of the calpastatin domain are able to bind the calmodulin-like domains of the large and of the small subunit of calpain, respectively (Takano *et al*., 1995). CaMLD binding and the degree of calpain inhibition have been demonstrated to be well correlated (Ma *et al*., 1994). Thus the tripartite subdomain structure of the inhibitory calpastatin domain (regions A, B and C) should contribute to the formation of a tight calpain-calpastatin

complex (Takano *et al*., 1995). Neither kininogen domain 2 nor the chicken cystatin-KD2 and stefin B-KD2 hybrids contain putative calmodulin-binding motifs. Accordingly, we found that only calpastatin domain 1 but not the cystatin-type inhibitors were able to interact with the S-CaMLD of the 21 kDa fragment.

Although the interaction of cystatins with papain-like enzymes involves three main contact regions, these are known to form a wedge-like contiguous contact area binding to spatially closely related primed and non-primed subsites of the enzymes (Bode *et al*., 1988). In contrast, the proposed tripartite nature of the calpastatin-calpain interaction would require simultaneous binding to the active site in domain IIa+IIb as well as to both CaMLDs (domain IV and domain VI) located quite far apart from it (Hosfield *et al*., 1999; Strobl *et al*., 2000). Solution of the three-dimensional structures of calpain complexes with calpastatin domains and kininogen domain 2 remains a challenge for future investigations.

Materials and Methods

Materials

All chemicals used were purchased from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Fluka (Buchs, Switzerland) and were of analytical grade. Restriction endonucleases and DNA modifying enzymes were from Roche Molecular Biochemicals (Mannheim, Germany) and New England Biolabs (Schwalbach, Germany). Human kininogen domain 2 obtained by limited proteolysis of human L-kininogen (Auerswald *et al*., 1996) was a generous gift from J. Brzin (Ljubljana, Slovenia). Recombinant human calpastatin domain 1 and calpastatin peptide B (calpain inhibitor peptide) were purchased from Calbiochem (Bad Soden, Germany) and Sigma, respectively. Papain (EC 3.4.22.2) from Boehringer (Mannheim, Germany) was repurified and active-site titrated with E-64 as described previously (Barrett and Kirschke, 1981; Machleidt *et al*., 1995). Human cathepsin L (EC 3.4.22.15) and human L-kininogen were purchased from Calbiochem. Fluorogenic substrates Z-Phe-Arg-AMC, Bz-Arg-AMC and Suc-Leu-Tyr-AMC were obtained from Bachem (Heidelberg, Germany). All reagents and materials for biomolecular interaction analysis were from Biacore AB (Uppsala, Sweden).

PCR Mutagenesis and Expression of ΔLeu 110 Chicken Cystatin-KD2Hybrids

Plasmids containing the genes for chicken cystatin r-cc and chicken cystatin-KD2 hybrids cc sc1/2-KD2, cc sc2/3-KD2 and cc sc1/2/3-KD2 (Auerswald *et al*., 1996) were used for construction of the corresponding ΔLeu 110-variants. The ΔLeu 110 chicken cystatin-KD2 hybrids were prepared by PCR mutagenesis using these plasmids as templates and the oligonucleotide primers CCΔL110FO(5'-TGCACTGCAGGTTGAAATCGGTC-3'), CCΔL110RE (5'-CCGCTCGAGTTTGATCTGGTTCAGCCACGG-3'), and SC3ΔL110RE (5'-CCGCTCGAGTTTAGCGATACGCAG-CTGGAT-3'). The PCR products were ligated into the expression vector pIN-III-ompA2 after digestion with *Eco*RI and *Bam*HI. Correct plasmids were selected after restriction analysis and DNA sequencing.

Synthesis, Cloning, Cassette Mutagenesis and Expression of Human StefinB and Stefin B-KD2 Hybrids

A synthetic gene for stefin B was designed using the GCG programme (HUSAR, Heidelberg, Germany) for editing DNA and amino acid sequences (Figure 2). The cDNA sequence for stefin B from GenBank (#LO3558) served as a scaffold for design. Whenever possible, codons present in highly expressed genes of *E.coli* (Kane, 1995) were selected to ensure good levels of expression. At the 5' end of the gene an *Eco*RI restriction site was added and at the 3' end two stop codons and a *Hin*dIII restriction site were included in order to make a direct cloning into pUC 18 (cloning vector) and pASK 40 (expression vector) possible. New restriction sites were introduced without alteration of the amino acid sequence. Unique sites already present in the DNA sequence were left undisrupted and repeated sites were eliminated to allow cassette mutagenesis. Synthesis was basically performed according to (Khorana, 1979; Ferretti *et al*., 1986). The gene was divided into 12 oligonucleotides, which were purchased from MWG-Biotech (Ebersberg, Germany). Briefly, 200 pmol of the internal oligonucleotides (SBwt-02, -03, -04, -05, -06, -07, -08, -09, -10, -11) were phosphorylated at their 5' ends. After phosphorylation, the internal oligonucleotides were mixed with 200 pmol of the external oligonucleotides (SBwt-01 and -12). This hybridisation mixture was incubated at 95 °C during 5min and cooled down overnight to room temperature. The internal nicks were subsequently ligated and the hybridisation product was purified by electrophoretic separation on a 2% low melting agarose gel. The DNA fragment was extracted from the gel and ligated in a molar ratio of 15:1 into a pUC 18 cloning vector previously digested with *Eco*RI and *Hin*dIII. Correct plasmids were selected after restriction analysis and DNA sequencing. The hybrid SBsc3-KD2 was synthesised in the same way as stefin B, replacing the oligos SBwt-09 and -10 by SBsc3-09 (5'-TGCGTGTTTTCC-AGGACATCCAGCTGCGTATCGCTTCCAA-3') and SBsc3–10 (5'-AAGGTCCTGTAGGTCGACGCATAGCGAAGGTTGATGTCT-3'). SBsc1-KD2 and SBsc1/3-KD2 were obtained by cassette mutagenesis. The new cassette sc1 (SBsc1-01: 5'-AATTCC-GCTGAAGGTCCGGTTGTTACCGCTCAGTACGACTCCCTGG-GTGCTGTTAGCGCTACCCAGCCGGCTACTGCA-3'; SBsc1-02: 5'-GGCGACTTCCAGGCCAACAATGGCGAGTCATGCTGA GGGACCCACGACAATCGCGATGGGTCGGCCGATG-3') carrying the sequence corresponding to the N-terminus of KD2 was ligated into the plasmids containing the correct sequences for stefin B and SBsc3-KD2 after removing the original cassette by *Eco*RI/*Pst*I restriction digestion. For periplasmic expression in *E. coli*, the genes obtained by synthesis or by cassette mutagenesis were subcloned into pASK40 (Skerra *et al*., 1991) *via* the *Eco*RI/*Hin*dIII restriction sites. Recombinant strains, obtained by transformation of JM 83 *E. coli* competent cells with the plasmids carrying the correct sequences of stefin B and stefin B-KD2 hybrids were cultured at 37 °C until $OD_{550\,nm}$ = 0.6–0.8 and induced, for heterologous expression, with 1 mM IPTG. The cells were grown 4 hours at 30 °C and harvested by centrifugation (4000 *g*, 25 min). Cell lysates were tested for expression by SDS-PAGE and Western blot analysis using polyclonal antibodies (rabbit) against human stefin B (DR Laboratories, Dießen) and POD-anti rabbit IgG (goat; Dianova, Hamburg).

Isolation and Purification of the Recombinant Hybrids

Periplasmic fractions were isolated according to (Dalböge *et al*., 1989) using a cold osmotic shock. Briefly, harvested cell pellets were washed with 1/5 of the culture volume of chilled 200 mM Tris/HCl, 20% sucrose, 100 mM EDTA (pH 9.0) and were shaken on ice for about 10 min. After centrifugation at 6000 *g* for 20min,

the pellets were resuspended in 1/5 of the culture volume of chilled 20 mM Tris/HCl (pH 7.5). Purification of the hybrids was achieved by affinity chromatography on a carboxymethyl-papain Sepharose column (Anastasi *et al*., 1983) followed by anion exchange chromatography on a Mono Q column using a FPLC system.

SDS-PAGE, HPLC, N-Terminal Amino Acid Sequencing and Mass Spectrometry

SDS-PAGE of the recombinant hybrids was performed in 15% polyacrylamide gels according to Laemmli (1970). The homogeneity of the purified hybrids was checked by reversed-phase HPLC. Two nmol of each sample were applied to a Jupiter C4-column (150 x 1 mm, Phänomenix, Aschaffenburg, Germany) and eluted with a linear gradient (0–60%) of acetonitrile in 0.1% TFA using a flow rate of 60 µl min^{-1}. The recombinant inhibitors were identified by N-terminal amino acid sequencing with a gas-phase sequenator 473A (Applied Biosystems, Weiterstadt, Germany) according to the instructions of the manufacturer. The molecular masses of the inhibitors were determined by mass spectrometry using a tandem quadrupole ionspray mass spectrometer API III (Sciex Thornhill, Ontario, Canada) basically as described elsewhere (Covey *et al*., 1988; Mann *et al*., 1989).

Inhibition Assays and Determination of Kinetic Constants

Inhibition of papain and cathepsin L was assayed at 30 °C and pH 5.5 with the fluorogenic substrate Z-Phe-Arg-NH-Mec as described in detail elsewhere (Machleidt *et al*., 1993, 1995). Inhibition of µ-calpain (5–10 nM) was measured in continuous fluorimetric assays at 12 °C (to slow down inactivation due to autolysis) in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.015% Brij-35, 100–150 µM $CaCl_2$, and 1mM dithiothreitol (freshly added) in a total volume of 500 µl using the fluorogenic substrate Suc-Leu-Tyr-AMC (250 µM). After a constant reaction rate was achieved (10–20 min), the inhibitors were added and the reaction was followed towards a stable equilibrium (15–90 min). The rate constants k_{on} and k_{off} for complex formation of cathepsin L, papain and calpain with L-kininogen, kininogen domain 2 and the hybrids were obtained by presteady-state analysis of the progress curves (Morrison, 1982). Equilibrium dissociation constants (K_i) were calculated from the rate constants ($K_i = k_{off}/k_{on}$) or, for some calpain inhibitors with very low affinity, measured directly in steady-state experiments, and were corrected for competition of the inhibitor with the substrate. The inhibitorily active concentrations of inhibitors were determined by titration with E-64-standardised papain (4 nM) using the substrate Bz-R-NH-Mec (10 µM) (Barrett and Kirschke, 1981).

Isolation of µ-Calpain from Human Erythrocytes

Human µ-calpain (EC 3.4.22.17) was isolated from erythrocytes following a protocol established in our laboratory (D. Gabrijelcic-Geiger and E. A. Auerswald, unpublished). Briefly, after lysis of erythrocytes and centrifugation, the supernatant was applied to a DEAE-Sepharose ion-exchange column. Bound fractions were eluted and further purified on Phenyl-Sepharose (separation of calpastatin from calpain). Enzymatically active fractions were applied to gel filtration on Superdex-200 and finally purified on Blue-Sepharose. The final product was homogeneous (80 kDa and 30 kDa subunits) and more than 95% pure as judged by SDS-PAGE and N-terminal sequence analysis. Average yields were about 2 mg of purified µ-calpain from 600 ml erythrocyte concentrate.

Cloning, Expression and Purification of a 21 kDa Calpain Small Subunit Fragment

A C-terminal fragment (21 kDa) comprising residues 86–268 (preceded by Met) was created by cloning the PCR product of the cDNA of the calpain 30 kDa subunit (kindly provided by K. Suzuki (Institute of Molecular and Cellular Bioscience, University of Tokyo, Japan) into a pET-22b(+) vector. Expression was achieved under the control of the T7 promotor in *E. coli* BL384, rendering yields of about 15 mg/l cell culture. The recombinant 21 kDa protein carrying a C-terminal His_6-Tag was isolated from the soluble fraction of cell lysates and purified by affinity chromatography on a Ni-NTA column. This procedure provided a >90% pure protein according to SDS-PAGE, N-terminal sequencing and mass spectrometry.

Biomolecular Interaction Analysis

Real-time biomolecular interaction analysis was performed using surface plasmon resonance technology in a BIAcore 2000 instrument (Biacore AB). The inhibitors were immobilised on CM5 chips *via* amine group coupling. After activation of the sensor chips with 35 µl 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*-[3-(diethylamino)propyl]carbodiimide (flow rate 5 µl min^{-1}), 35 µl of the inhibitor solutions (25–100 µg ml^{-1} in 10 mM sodium acetate, pH 4.2) were injected, followed by 35 µl of 1.0 M ethanolamine (pH 8.5) for deactivation of unreacted groups. HBS buffer (Biacore) was used as running buffer throughout the coupling procedure. After coupling, the chip was washed with 3.0 M NaCl, 0.1 M NaOH to remove non-covalently bound ligands. Binding experiments were performed at 25 °C in 50 mM Tris/HCl, 100mM NaCl, 0.015%Brij35, pH7.5, as running buffer by injecting the analyte solutions (5–100 µg ml^{-1}) over 10min with a flow rate of 5 µl min^{-1}. The surface was regenerated between cycles by injecting 25 µl of 3.0 M NaCl, 0.1 M NaOH.

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