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A Novel Class of Small Functional Peptides that Bind and Inhibit Human α-Thrombin Isolated by mRNA Display

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Summary

Here we report the in vitro selection of novel small peptide motifs that bind to human α-thrombin. We have applied mRNA display to select for thrombin binding peptides from an unbiased library of 1.2×10^{11} different 35-mer peptides, each containing a random sequence of 15 amino acids. Two clones showed binding affinities ranging from 166 to 520 nM. A conserved motif of four amino acids, DPGR, was identified. Clot formation of human plasma is inhibited by the selected clones, and they downregulate the thrombin-mediated activation of protein C. The identified peptide motifs do not share primary sequence similarities to any of the known natural thrombin binding motifs. As new inhibitors for human thrombin open interesting possibilities in thrombosis research, our newly identified peptides may provide further insights into this field of investigation and may be possible candidates for the development of new anti-thrombotic agents.

Introduction

In vitro display techniques are powerful tools to identify polypeptides with desired features and functionalities from random sequence libraries. There are now a number of methodologies available that allow the physical and unambiguous connection of protein primary sequences with their encoding mRNAs. Techniques such as phage [1, 2], ribosome [3–5], and mRNA display [6–10] allow researchers to perform protein in vitro evolution experiments using libraries of peptide and protein sequences. With respect to library complexity and simplicity and stability of the connection of genotype and phenotype, mRNA display is among the most powerful approaches. The technique benefits from the covalent linkage between the mRNA and its encoded polypeptide, mediated by the antibiotic puromycin. After enriching functional molecules of displayed peptides or proteins, the sequence information can easily be retrieved by analyzing the attached mRNA. Using this approach, the frequency of the occurrence of ATP [11] or streptavidin binding [12] protein motifs contained in a functionally unbiased protein library has been determined. These studies indicated that functional proteins can be identified in such libraries by entirely stochastic means.

We were interested in the de novo selection of peptides that recognize a protein target that is bound and affected by a diverse set of natural peptides at various epitopes. One prominent example is human α-thrombin, a key enzyme in blood coagulation [13] that modulates procoagulant as well as anticoagulant processes. First, the serine protease thrombin converts fibrinogen into fibrin, which finally leads to clot formation, and second, it regulates its own production level by activation of factors V, VIII, and XI [14–16]. The structure and surface of thrombin has been intensively studied, and in addition to the active site cleft, two main positively charged epitopes termed the anion binding exosites have been identified: (1) the fibrinogen recognition exosite (FRE or exosite-1) and (2) the heparin binding exosite (exosite-2) [17]. Many natural thrombin inhibitors like heparin, hirudin [18–20], hirugen [21], haemadin [22, 23], heparin cofactor II [24], and antithrombin III [25, 26], which bind to either or both sites on the thrombin surface, have been identified and characterized and were subsequently engineered [27]. They play an important role as anticoagulants in a large variety of clinical applications. Of high clinical relevance is heparin, a polysulfated glycan that promotes the binding of heparin cofactor II and antithrombin III. Another exosite-2 binding protein is the platelet glycoprotein Ibα [30], but its biological role has not been fully determined to date. Thrombomodulin binds to human thrombin on the surface of endothelial cells and by the formation of the complex protein C activation [28, 29] is accelerated. Table 1 summarizes different proteins and SELEX-derived nucleic acids that bind to α-thrombin.

These examples indicate that human α-thrombin is a protein recognized and affected by a wide variety of different protein motifs. We have used in vitro selection of a library of mRNA-displayed peptides, restricted in length but unbiased in their primary sequence, to isolate functional sequences that bind to human α-thrombin. The mRNA display molecules used in the selection comprised a 15 amino acid long randomized region comparable to the length of identified protein motifs interacting with α-thrombin (see Table 1). One aspect we were particularly interested in was the question regarding the frequency of the occurrence of thrombin binding peptides in this library. Our study also addresses the question of how selected sequences compare with natural ones with respect to primary sequence, the occurrence of binding motifs, binding affinity, epitope selection, and inhibitory activity. The selection of novel peptide ligands

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Table 1. Binding Motifs of Different Ligands for α-Thrombin

Name	Interacting Sequence	Reference
Antithrombin III	391-AGRSLNPNRVTFKA-404	[27]
DNA aptamer	1-GGTTGGTGTGGTTGG-15	[40, 56]
Fibrinogen		
Fibrinopeptide Aα	52-WPFCSDEDWNY-62	
Fibrinopeptide Bβ	56-APSLRPAPPPISGGGYR-72	[51, 52]
Haemadin	46-EFEEFEIDEEEK-57	[22]
Heparin cofactor II	54-NDWIPEGEEDDDYLDLEKIFSEDDDYI-75	[24, 53]
Hirudin	54-DFEEIPEEYLQ-65	[19, 20]
Platelet glycoprotein Ibα	272-DTDLYDYYPEE-282	[57, 58]
RNA aptamer	1-UCCGGAUCGAAGUUAGUAGGCGGA-24	[36]
Thrombomodulin	415-LDDGFICTDIDECEN-429	[42, 51, 54, 55]

Numbers refer to the positions of residues within epitopes that contact α-thrombin.

to human α-thrombin modulating its diverse interactions should provide further insight into the multifunctional role of the protease.

Results and Discussion

Library Design

We designed and synthesized a DNA library encoding a 35-mer amino acid sequence containing 15 randomized amino acid positions [31] (Figure 1A). Similar to the work of Baggio et al. [32] and Cujec et al. [33], the codon triplets for each amino acid were not subject to any bias in terms of imposing α helix or β sheet patterning. After the translation initiation site, we introduced TGT as a codon for a fixed cysteine residue, allowing the possibility of forming conformational constraints via disulfide bridges. The random region was designed for low stop codon frequency and increased cysteine probability (1 Cys within the random region). To facilitate purification on Ni^{2+}-NTA agarose, a 6× histidine-tag (his6) was inserted. By introducing the his6 tag downstream of the randomized region, we only purified molecules that did not contain stop codons or frame shifts. The DNA library was transcribed using T7 RNA polymerase, and the obtained RNA was linked to a synthetic DNA oligonucleotide bearing the puromycin moiety at its 3′ end. The mRNAs linked to puromycin were in vitro translated, and approximately 1.2×10^{11} different peptide displaying molecules were obtained after purification on oligo-dT cellulose, Ni-NTA agarose, and reverse transcription. A randomized region of 15 amino acids theoretically yields 3×10^{19} (20^{15}) unique sequences. This sequence space is only partially covered by the synthesized library but still resembles the potential repertoire of the human antibody complexity.

Selection

Binders to human α-thrombin were enriched after 10 cycles of selection and amplification. For the selection a cyanogenbromide-activated sepharose matrix was derivatized with human α-thrombin. Using this method, the protein is statistically coupled to the matrix, which allows screening of the entire exposed surface. The mRNA-display library (11 pmol) was then incubated with the immobilized thrombin. After appropriate washes (10 column volumes [CV] in the first four rounds), the retained molecules were eluted under denaturing conditions. PCR amplification of these molecules yielded the starting material for the next round of selection. In round 3, a preselection against a nonderivatized matrix was introduced to eliminate possible matrix binders. The wash volume was slightly increased in round 5 to 15 CV, and when the binding reached 42% in this fifth round (Figure 1B), the stringency was increased to 25 CV in the succeeding cycle followed by a decrease in binding to approximately 18%. As expected, binding went further down to approximately 10% with 200 CV of wash volume in cycle 6. In the subsequent rounds of selection, binding rose again and reached 25% in round 10. The eluted molecules were then amplified by PCR and consecutively cloned and sequenced. Seventy-two clones were randomly chosen for analysis. The obtained clones resulted in 45 different sequences (Figure 1C), but more than 60% contained a highly conserved four amino acid sequence motif, DPGR. As shown in Figure 1C, five sequences appeared in duplicates and eight other sequences were identical, adding four with only point mutations. The DPGR motif could be found at almost every position of the random sequence, although a position closer to the C-terminal end seems to be preferred. From this analysis we conclude that this motif is obviously crucial for the recognition and binding between the clones and α-thrombin. Furthermore, 24 different sequences (42%) contained a second cysteine residue. Interestingly, none of the selected sequences showed any primary sequence similarity to the natural thrombin binding motifs (Table 1).

Binding Studies

To characterize the obtained sequences, two different clones, designated as T10-11 (sequence of the random region: ERNYNDFCDPGRVGL) and T10-39 (FFDRYDS ARDPGRLL) from the enriched round 10 pool were chosen for further studies. As a control sequence, clone T10-35 (WQRCGMAEFIWHFQW) was chosen from the selected pool, and from the unselected starting pool a sequence named P-11 (VRIAASRISTNNKYF) was selected. T10-11 and T10-39 both contain the DPGR motif, and T10-11 additionally features a second internal cysteine residue. Clone T10-35 contains neither the DPGR motif at any position nor the cysteine residue. First, the RNA of each sequence was prepared and directly (without ligation to the puromycin linker) subjected to in vitro translation in the presence of ^{35}S-labeled methio-

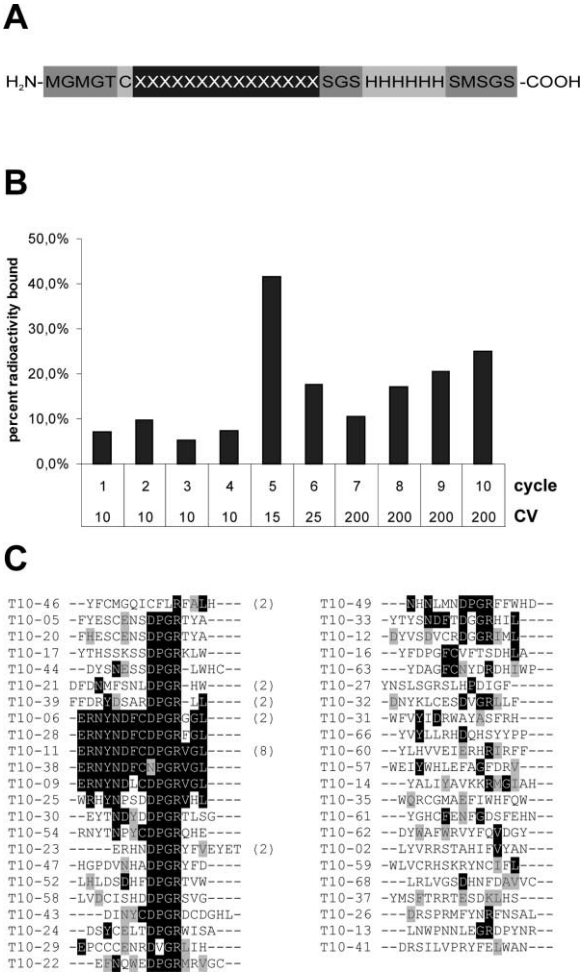

Figure 1. Library Design and Course of Selection

(A) Schematic drawing of the library design. The randomized region is shaded black where X represents an amino acid residue. Constant regions are shaded gray, and the fixed cysteine residue and the histidine-tag are shaded light gray.

(B) Course of selection. Bars represent the percentage of radioactivity that bound to the column matrix in each cycle. Below, the number of each cycle is given and the volume of wash buffer in column volumes (CV) used in each cycle.

(C) Analysis of the obtained sequences from round 10 (only the 15 randomized amino acids are shown). More than 60% of the clones contain the 4 amino acid DPGR motif. Numbers in brackets indicate how often clones were found.

nine to gain the free peptide. According to Barrick et al. [34], the relative binding of the clones were determined and percentages of bound free peptide were calculated by phosphorimager integration (Figure 2A). To investigate whether the his6 tag is involved in the binding, sequences lacking the tag (termed His^-) were prepared and treated the same way as described above. The starting pool and the control sequence P-11 do not bind to the thrombin-derivatized matrix, whereas the selected clones T10-11 and T10-39 and the his6 truncated versions show retention on the thrombin sepharose ranging from 50% to 85% based on a normalized level regarding the radioactivity of the free peptide. Sequence T10-35, which derived from the enriched pool, shows very weak binding to the matrix. Removal of the his6 tag has only minor effects and leads to a decrease in the relative binding activity by approximately 15%. Although the elimination of the his6 tag does not decisively influence the binding, contribution of the constant amino acid regions cannot be completely excluded.

Competition studies under the described conditions were performed adding free thrombin to the binding assays. Increasing free thrombin reduces the binding of the free peptide to the matrix (data not shown). The apparent inhibition constants (K_i) for thrombin were determined to be 295 nM and 236 nM for the T10-11 and the T10-11 His^- construct, respectively. Thrombin shows K_i's of 228 nM and 457 nM for the T10-39 and the T10-39 His^- constructs, respectively. The relatively high K_i of ~450 nM for the T10-39 His^- construct, compared to ~250 nM for the others, suggests strong binding of this free peptide to the matrix because high levels of free thrombin are necessary to compete the binding. To determine the dissociation constants of both clones, the 29-mer peptides of the T10-11 His^- and T10-39 His^- constructs were custom synthesized and coupled to surface plasmon resonance sensor chips. Thrombin was used as analyte at increasing concentrations (1–2500 nM). Dissociation constants were then calculated by the BIAevaluation software using steady-state affinity curve fits (Figures 2B and 2C). K_d values of 520 ± 6 nM for T10-11 and 166 ± 18 nM for T10-39 were determined. Although clone T10-39 is not the most abundant one, it shows better binding to thrombin, which is in accordance to the result of the relative binding studies and the included determination of the apparent inhibition constant. It further suggests that the formation of a disulfide bridge is not crucial for binding, because clone T10-39, in contrast to clone T10-11, does not contain a second cysteine. As a control we used human neutrophil elastase, which is related to the protease thrombin, and no binding to the immobilized selected peptides was detected under these conditions up to concentrations of 5 μM elastase (data not shown).

Epitope Mapping

We next sought to narrow down which epitope of thrombin is recognized by the small peptides. We performed competition binding studies in solution by using hirudin, hirugen (amino acids 54–65 of hirudin in the sulfated and nonsulfated modification), and antithrombin III among the natural thrombin binding peptides shown in Table 1 as representatives of exosite-1 and -2 binders. We also investigated the ability of the SELEX-derived DNA- [35] and the 24 nucleotide RNA aptamer 16.24 [36] to compete with the peptide aptamers for thrombin binding. Peptides T10-11 and T10-39 were immobilized on the flow-cell and incubated with 50 nM thrombin, and increasing concentrations of the different thrombin binders were added (for Supplemental Data, contact *Chemistry and Biology* at chembiol@cell.com). The obtained responses were normalized to the binding without competitor and curves fitted using the program Origin 6.1 (OriginLab Corporation, Northampton, MA). Apparent competition constants (K_i's) are listed in Table 2. Hirudin showed a K_i of ~550 nM, whereas competition

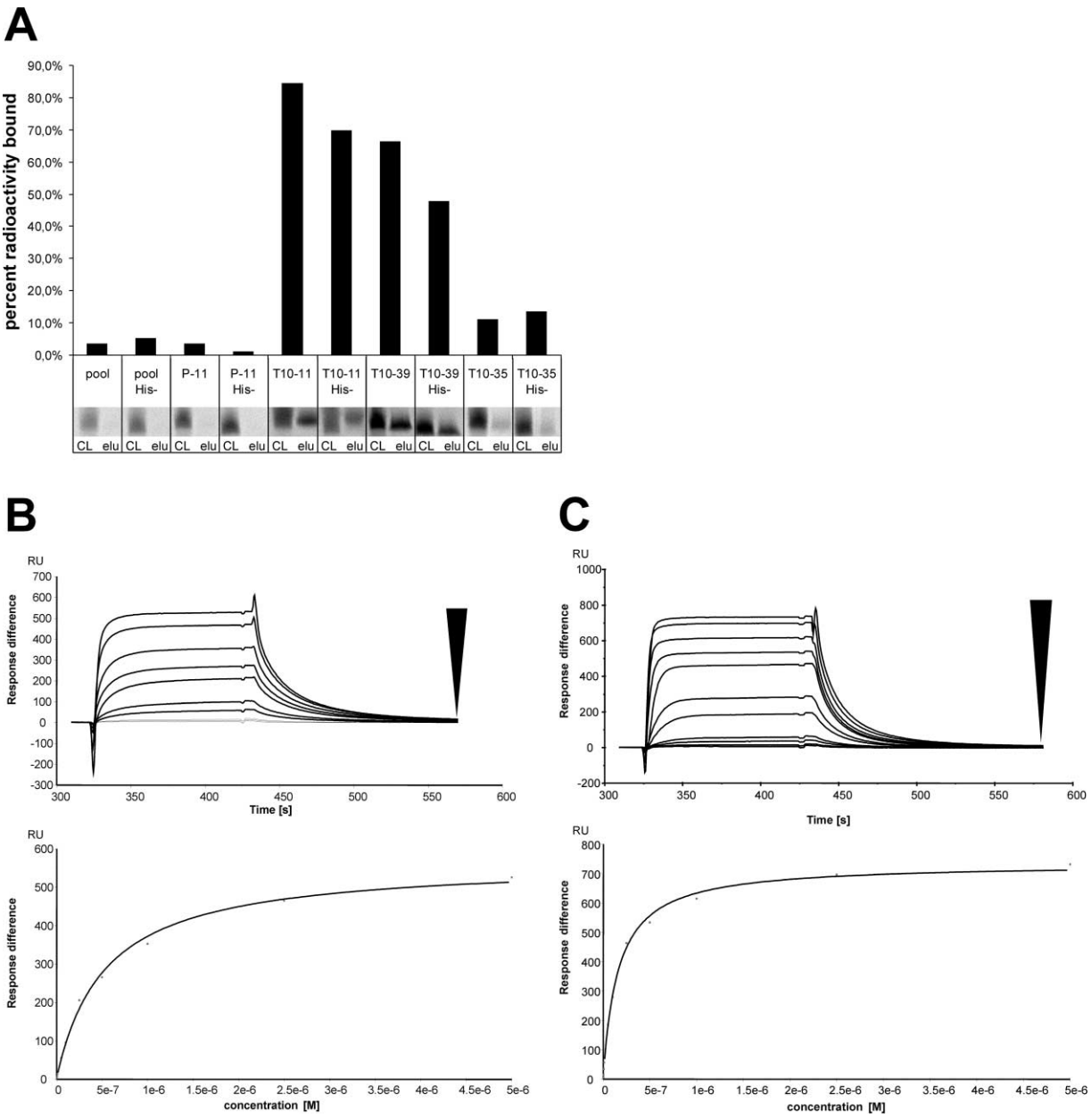

Figure 2. Binding Studies and K_d Determination

(A) Bars illustrate the binding of the free ^{35}S-labeled peptides to the thrombin-derivatized matrix. Gels of the crude lysate (CL) and the eluted peptide (elu) are shown below each construct.

(B) and (C) illustrate the K_d determination of clone T10-11 and T10-39, respectively. In the upper part, the binding curves (time versus response difference in response units [RU]) of thrombin on immobilized peptides are shown. The lower level shows the steady-state curve fits (concentration of peptides against RU). The arrowheads indicate the increasing thrombin concentrations (1–2500 nM). The K_d values of T10-11 and T10-39 were determined to be 520 nM and 166 nM, respectively.

by the sulfated hirugen is 1.4- to 1.6-fold weaker for T10-11 and T10-39, respectively, and 3.3- to 5.9-fold weaker for the nonsulfated peptide (Table 2). The K_i of antithrombin III is 8.1- to 9-fold higher than that of hirudin. Interestingly, the DNA aptamer can compete with the selected peptides much more efficiently than hirudin, although it binds to the thrombin exosite with a K_d of only 200 nM [35], which is a considerably weaker affinity than that of hirudin. The same trend is observed in another competition assay in which immobilized thrombomodulin and thrombin were used as analytes. Here again the DNA aptamer blocked the interaction of those two proteins with a lower K_i than hirudin (data not shown). The RNA aptamer exhibited a similar K_i as the DNA aptamer, although competition with heparin suggested that this RNA binds the electropositive heparin binding site of thrombin with higher preference than the exosite-1 [36]. At present, we cannot explain why both

Table 2. Apparent Competition Constants of Various Thrombin Binders with Immobilized T10-11 and T10-39

	Apparent Competition Constants (nM)	
Inhibitor	T10-11	T10-39
Hirudin	524 ± 29	575 ± 16
Hirugen	1721 ± 133	3413 ± 299
Hirugen (SO_4)	743 ± 121	933 ± 211
DNA aptamer	47 ± 5	57 ± 3
RNA aptamer	49 ± 4	59 ± 3
Antithrombin III	4269 ± 366	5214 ± 296

The table shows competition constants determined by surface plasmon resonance. 50 nM thrombin was incubated with increasing concentrations of thrombin binders. The obtained curves (see Supplemental Figure S1) were fitted in Origin 6.1 to obtain the corresponding apparent binding competition constants (K_i's).

aptamers compete with similar activity with our selected peptides for thrombin binding although they bind to different surfaces of the protein. Their activity is specific because random nucleic acids show no effect in the same experimental setup (data not shown). It is possible that the comparable K_i's reflect the marked differences in the K_d of both aptamers. While the K_d of the DNA aptamer is reported to be around 200 nM [35], the 16.24 RNA aptamer binds thrombin with a K_d of 9 nM [36]. Furthermore, it has been reported that serpins, when complexed to exosite-2, allosterically disorganize exosite-1 [37, 38]. It is possible that the binding of 16.24 does the same, thereby affecting the binding of the selected peptides at a distal site.

In order to interpret the other competition data shown in Table 2, we evaluated structural data available for the various thrombin binding targets used as competitors in Table 2. These data are summarized in Figure 3. Figure 3A shows thrombin without the complexed ligands, and exosite-1 residues highlighted in orange. Figure 3B shows thrombin in exactly the same orientation as in Figure 3A with hirudin (red) [39], the DNA aptamer (blue) [40, 41], and thrombomodulin (yellow) [42] superimposed. While all three ligands make contacts to exosite-1 residues, it appears that the degree to which the surface of exosite-1 is utilized for binding is different for each ligand. The surface within exosite-1 in which contacts of each of the three ligands overlap is rather small. Apart from these overlapping exosite-1 contacts, the binding sites of the three ligands vary considerably. It is evident that the binding site of the DNA aptamer, which shows enhanced competition in our studies, is located fairly apart from that of the other two ligands.

Taken together, these data suggest that our selected peptide aptamers target a site on thrombin that is more closely matched by the DNA aptamer than by hirudin, thrombomodulin, or antithrombin III. As hirudin and the DNA aptamer both have contacts with residues within the hirudin/fibrinogen binding site (exosite-1), but the DNA aptamer is a more active competitor, it is likely that the small peptides interact with parts of the same surface that is also contacted by the DNA aptamer. Whether this interaction surface also includes exosite-1 residues cannot be said with certainty. However, we found that T10-11 and T10-39 show significantly reduced (3- to 9-fold weaker) binding to γ-thrombin, which lacks the exosite-1 (data not shown), indicating that exosite-1 residues participate in binding of these peptides to some extent.

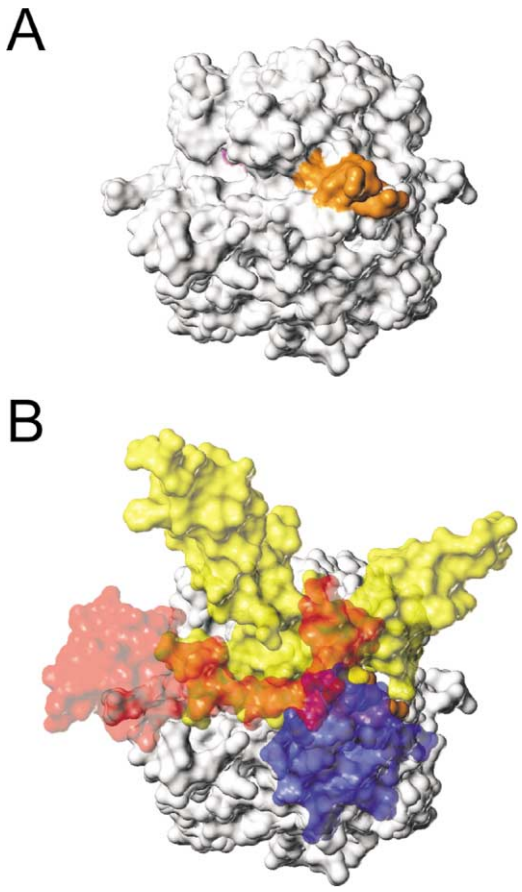

Figure 3. Binding of Inhibitors to Thrombin

(A) The figure shows the structure of α-thrombin (gray) with the active site in magenta and exosite-1 residues in orange.

(B) Thrombin in exactly the same orientation including the superimposed structures complexed hirudin (red), thrombomodulin (yellow), and the DNA aptamer (blue). Structures were obtained from the available data of 4htc [17, 19, 39], 1dx5 [42], and 1hap [40] and fitted using the program package Sybyl 6.8 on a Silicon Graphics Octane workstation. All three ligands share a small surface of overlapping contacts on the thrombin surface.

SPOT Analysis

After we have shown the binding of the selected peptides, we were interested in the minimal motifs and which amino acids are necessary for the interaction. The peptides of clone T10-11 and T10-39 were subject to further analysis using the SPOT technique (reviewed in Reineke et al. [43] and references therein). Peptides of 13 amino acids length were synthesized on a cellulose membrane, each sequence overlapping one amino acid of the original sequence. Therefore, in total 17 spots of 13-mers that correspond to residues 1–13, 2–14, 3–15, … 17–29, spanning the entire 29 amino acid sequence, were synthesized. The membrane was then treated as described in the Experimental Procedures section and

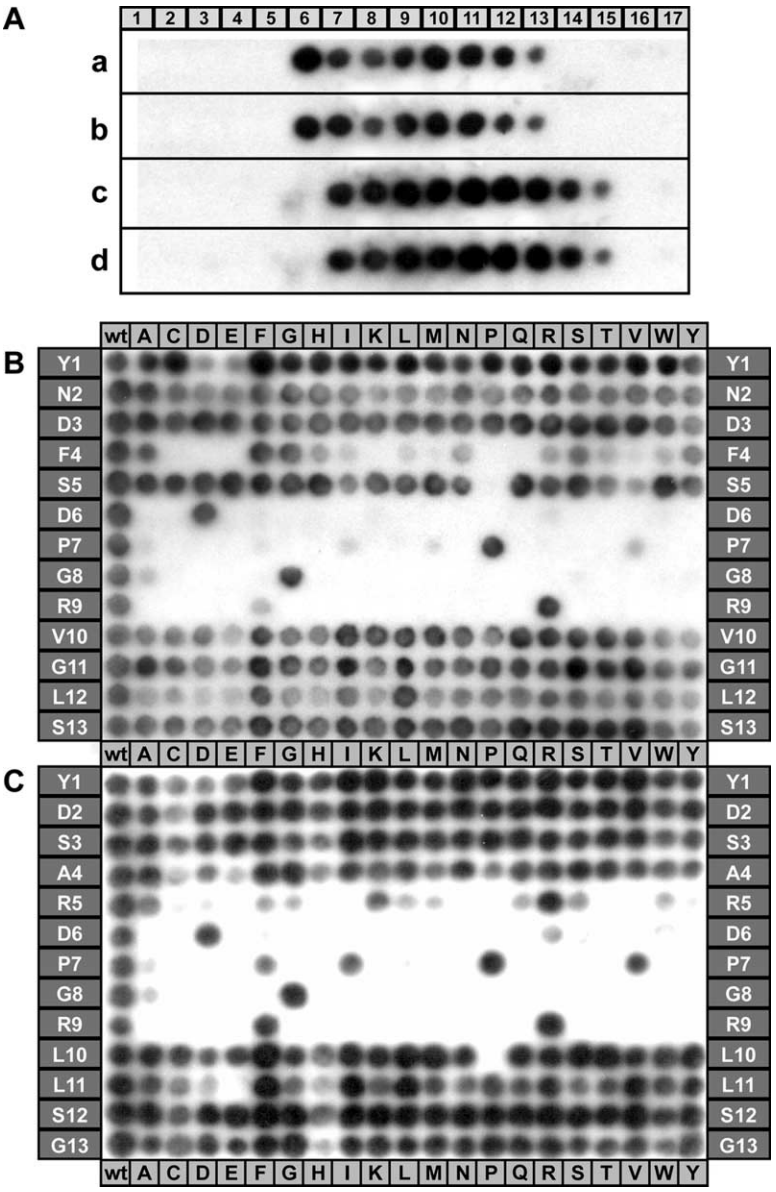

Figure 4. SPOT Analysis

(A) Overlapping peptide scans of clone T10-11 (rows a and b) and clone T10-39 (rows c and d). Peptides were prepared on cellulose membranes as 13-mers (numbers indicate each of the 17 different single sequences) covering the whole 29 amino acids of the single clones. Rows a and c indicate the original sequence, and rows b and d illustrate the binding pattern with cysteine exchanged against serine.

(B) Substitution scan of clone T10-11 and (C) of clone T10-39. Each minimal motif is given in the vertical panels. Each row starts with the wild-type (wt) sequence and the next spots indicate amino acid exchanges against the amino acids depicted in the horizontal panels.

documented on X-ray films (Figure 4A). Analysis of the overlapping regions revealed that in clone T10-11 (*MGMGTC*ERNYNDFCDPGRVGL*SGSSMSGS*), the minimal sequence required for thrombin binding is a 7 amino acid fragment (underlined; constant amino acids are shown in italics) (Figure 4A, a). For clone T10-39 (*MGMGTC*FFDRYDSARDPGRLL*SGSSMSGS*), this fragment could be determined to be as small as 6 amino acids (underlined) (Figure 4A, c). Both minimal motifs contain the amino acid motif DPGR, which reflects the observation from the sequence analysis. Exchanges of the cysteine residues in both clones against serine do not alter the binding pattern (cf. Figure 4A, b/d), which suggests that the formation of a disulfide bond is not necessary for thrombin recognition. This finding is further supported because cyclization of the membrane bound peptides of clone T10-11 shows no difference to the pattern obtained from the linear peptides (data not shown). Other SPOT peptides that were sequentially truncated from the N- or the C-terminal end or both were synthesized in parallel and spotted individually on a cellulose membrane. For this "length scan," the minimal motif plus the flanking amino acids of each clone were spotted on a cellulose membrane. Analysis of the binding pattern revealed that the length of the peptide that still binds to α-thrombin could be cut down from 13 amino acids to only the four amino acids of the DPGR motif and one flanking amino acid at either side (data not shown). To further investigate which amino acids are crucial for binding, we performed a "substitution scan" [44] of clones T10-11 and T10-39. The sequence containing the exchanged serine residue was chosen for clone T10-11 because cysteine might cause oxidative problems in the setup. For the substitution scan, again the minimal motif of each clone was spotted on a cellulose membrane and each single amino acid was then sequentially substituted against all 19 other ones. The obtained binding pattern for clone T10-11 (Figure 4B)

reveals that the first three C-terminal and the last four N-terminal amino acids of T10-11 can be exchanged against almost any other of the 20, resulting in only minor differences in the binding level. In contrast, the phenylalanine residue at position 4 shows exchange ability only with few other residues like alanine, glycine, histidine, asparagine, serine, or tyrosine. Interestingly, position 5 (Ser) loses its binding capability when it is exchanged against proline, suggesting that proline completely breaks the structure of the peptide. Clone T10-39 (Figure 4C) shows a very similar binding pattern. The N- and C-terminal amino acids are relatively insensitive toward changes. The arginine residue at position 6 can only be substituted by a lysine, which can be explained because of the similar structure of the side chain. The highly conserved DPGR motif (Figure 1C) is very sensitive to exchanges, and binding is only restored if amino acids are exchanged against each other. To reestablish binding, aspartic acid has to be exchanged against aspartic acid, proline against proline, glycine against glycine, and finally arginine against arginine. Within those four amino acids, aspartic acid can be partially substituted by arginine and the proline residue by the hydrophobic amino acids phenylalanine, isoleucine, and valine. Glycine is crucial at its position and the arginine residue can be exchanged against phenylalanine. Furthermore, in the two leucine residues following the DPGR motif in clone T10-39, two gaps appear. The first leucine shows no binding any more if it is substituted by proline, the second if it is substituted by glutamic acid. It can be seen that the conserved DPGR motif is very susceptible to substitutions, which indicates that those four amino acids are crucial for thrombin recognition.

The obtained minimal sequences of the two clones were custom synthesized, comprising either 7 or 8 amino acids. Sequences Ac-FSDPGRVG-NH_2 (designated as M8T10-11) and Ac-SDPGRVG-NH_2 (M7T10-11) from clone T10-11 and the sequence Ac-ARDPGRLLS-NH_2 (M8T10-39) from clone T10-39 were synthesized either regularly N-terminal free/C-terminally acetylated (Ac) or N-terminal free/C-terminally biotinylated bearing two β-alanine residues between the biotin moiety and the first amino acid. The biotinylated peptides were used in surface plasmon resonance studies on streptavidin sensor chips. Unfortunately, no significant interaction between the minimal sequences and thrombin could be detected. It is known that low-affinity interactions can be detected by the SPOT technique because of a very high local concentration of the peptide and the signal amplification by the horseradish peroxidase [45]. It is also possible that the streptavidin surface of the sensor chip may interfere with the binding event. In any case, this result shows that the minimal motif alone is necessary but not sufficient for high-affinity interaction with thrombin. Flanking amino acids are required to increase the binding affinity between the selected peptides and their target.

Functional Studies

As thrombin comprises a large variety of functions, we investigated how its activity is influenced by the selected peptides. Thrombin activity was measured using a chromogenic substrate, S-2238. Upon the thrombin-catalyzed cleavage of the substrate, a nitroaniline derivative is released, which can be detected by measuring the optical density (OD). Therefore, thrombin was mixed with the peptides in varying concentrations, and the thrombin activity analyzed by the liberation of the chromophore (Figure 5A). The peptides T10-11 and T10-39 as well as the control peptide P-11 showed no influence on the thrombin activity. Neither did the constructs M8T10-11, M7T10-11, M8T10-39, the DNA aptamer, and antithrombin III (data not shown). Only hirudin as an active site binder blocked the enzymatic activity in this assay, as expected. This indicates that the interaction of the peptides with the thrombin molecule does not interfere with the active site. To analyze whether the small peptides are able to inhibit clot formation, measurements with a coagulometer using human plasma were performed (Figure 5B). Standard clotting time of human plasma with added thrombin was ~15 s. Increasing concentrations (0.2 to 80 μM) of the small peptides were added and thrombin clotting times determined. Both T10-11 and T10-39 show significant elongation of the clotting time up to 5-fold at a concentration of 80 μM, whereas the short constructs M8T10-11 and M7T10-11 do not decisively influence the clotting time (data not shown). Only M8T10-39 doubles the time until clot formation at 80 μM (data not shown). The control sequence P-11 shows no effect. A recently selected peptide that binds coagulation factor VIIa [46] shows a similar effect as it prolongs the clotting time up to 5-fold at a concentration of 100 μM. We conclude that the selected peptide sequences can influence thrombin activity by blocking the interaction between fibrinogen and thrombin. In comparison with hirudin or the DNA aptamer, which are able to completely inhibit clotting of plasma, our selected peptides perform less well. However, since the selection protocol was designed for binding, it is interesting to note that our peptides interact at the same epitope as the DNA aptamer.

To analyze whether the selected peptides alter the formation of the complex between thrombin and thrombomodulin (TM), which then activates protein C, we performed inhibition studies (Figure 5C). Thrombin and TM were premixed and the peptides added at increasing concentrations. Protein C was included and the production of activated protein C was measured using a chromogenic substrate, S-2366. We determined that both of the selected peptides decrease the protein C activation to 10% of the original level at a concentration of 100 μM. The apparent inhibition constant (K_i) of protein C activation by the peptides were determined to be 5 and 8 μM for T10-11 and T10-39, respectively. A previously rationally designed peptide inhibiting the interaction between thrombin and thrombomodulin revealed an apparent K_i of only 94 μM [47]. Compared to our selected peptides, the DNA aptamer showed nearly the same activity in blocking protein C activation ($K_i = 4$ μM). This finding is in accordance with the epitope-mapping data, which suggest that the selected peptides contact thrombin at a similar site as the DNA aptamer. The control peptide P-11 seems to have some unspecific effect at high concentrations but does not decisively influence

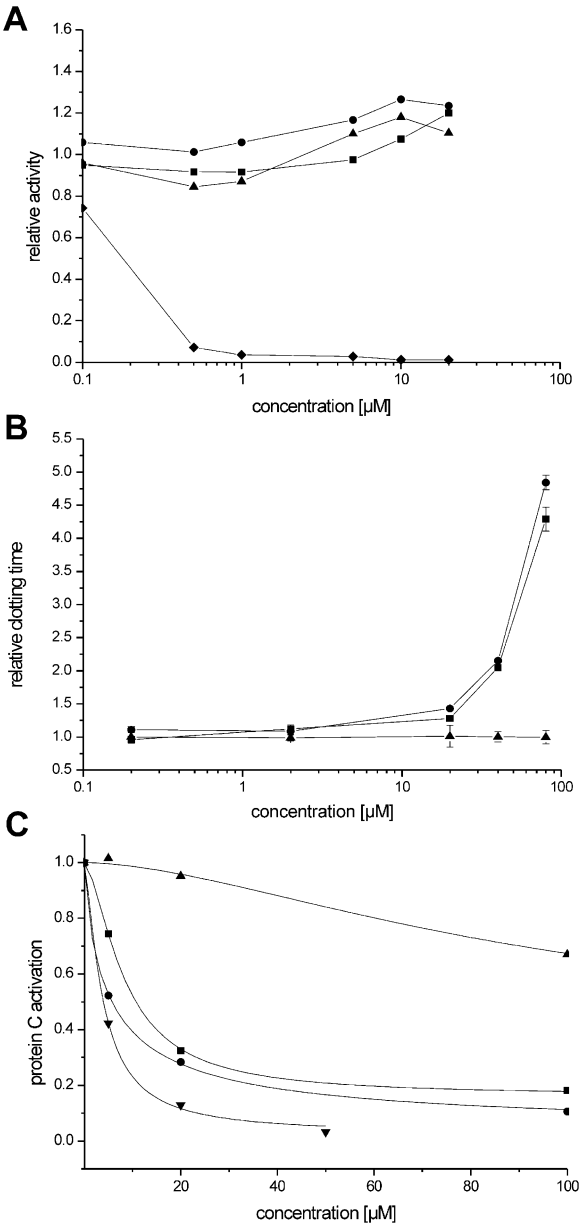

Figure 5. Functional Studies

Used peptides are T10-11 (filled circle), T10-39 (filled square), and the control sequence P-11 (filled triangle).

(A) Proteolytic activity assay of thrombin, measured by liberation of the chromophore from chromogenic substrate S-2238. Peptide concentration is plotted against relative activity. As a positive control, hirudin (filled diamond) was included in the analysis.

(B) Clotting analysis of human plasma. Peptide concentration is plotted against the clotting time. At a concentration of peptides T10-11 and T10-39 of 80 μM, clotting time is elongated almost 5-fold. P-11 shows no effect.

(C) Protein C activation. Upon binding to thrombomodulin, thrombin activates protein C. Peptide concentration is plotted against the relative initial velocity (first 6 min) of the protein C activation, measured by liberation of the chromophore from chromogenic substrate S-2366. T10-11 and T10-39 show strong effects by decreasing the production level of protein C; P-11 does not significantly influence this reaction. As a positive control, the DNA aptamer (filled inverted triangle) was used in this assay showing slightly higher efficacy in inhibiting protein C activation than the selected peptides.

the protein C activation. The K_i of P-11, which is difficult to determine, was calculated to be at least 150 μM. These observations are in accordance with the proposed binding site of T10-11 and T10-39 at or in close proximity of exosite-1.

Significance

Thrombin plays an important role in various reactions within the blood clotting cascade. In this work we present the evolution and identification of thrombin binding peptides out of a entirely randomized peptide library. We show that the selection of functional amino acid motifs out of a random library comprising 15 amino acids in length with a diversity of $\sim 1.2 \times 10^{11}$, which reflects the human antibody repertoire, successfully leads to functional sequences. The selection resulted in a highly conserved amino acid motif consisting of only four amino acids, namely DPGR. The evolved binding motifs, however, show no similarity with other naturally occurring protein domains binding to human α-thrombin. Thus, in vitro selection strategies like mRNA display allow the identification of novel, nonnatural functional sequences that can be used to investigate how protein-protein interactions are organized. Our data are in accordance with the notion that the selected peptides target thrombin at a similar site as the DNA aptamer. Natural thrombin binders can be found to contact either epitope of the thrombin molecule. Although the binding affinity is not as high as of natural proteins, we could demonstrate that the selected sequences are able to influence the thrombin activity by either prolonging the clotting time of human plasma or by downregulating protein C activation. As in vitro selection methods are open to find solutions to almost every desired functionality, this study may contribute to the identification of new and potent regulators of the blood coagulation cascade.

Experimental Procedures

Library Preparation

Approximately 4 nmol of the 99-mer ssDNA template (5′-CTATTTACAATTACAATGGGTATGGGTACCTGT5675675675675675675675675675675675675675TCCGGATCCCATCATCATCAC-3′) encoding 15 random amino acids and containing a 5′ KpnI and 3′ BamHI restriction site (underlined) and encoding the amino acid sequence H_2N-MGMGTC$(X)_{15}$SGSHHHHHHSMSGS-CO_2H (X is a random amino acid, cf. Figure 1A) was synthesized by standard phosphoramidite oligonucleotide synthesis. The numbers 5, 6, and 7 in the DNA sequence represent phosphoramidite mixtures that were optimized for each codon position using a computer algorithm [48]. According to Pollard et al. [49], the phosphoramidites of A, G, C, and T were dissolved in acetonitrile and then mixed for each codon position as follows: 5, A 19.2%, G 23.4%, C 23.4%, T 34.0%; 6, A 33.2%, G 32.9%, C 13.5%, T 20.4%; and 7, A 0.00%, G 31.0%, C 34.5%, T 34.5%. PCR amplification of the synthesized oligomer using the following 55-mer 5′-primer containing the T7 RNA polymerase promotor (underlined) and sequences required for translation initiation (5′-TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAATGGGTATGGGTA-3′) and the 41-mer 3′-primer containing the sequence for the 6× histidine tag and ligation sites (5′-ACTGCCGCTCATACTATGGTGGTGATGATGATGGGATCCGG-3′) yielded the dsDNA template that was used for transcription with T7 RNA polymerase (Stratagene, Amsterdam, The Netherlands). The obtained RNA was ligated to the DNA oligo bearing the puromycin moiety at its 3′ end via splint-mediated blunt end ligation using T4

DNA ligase and T4 RNA ligase (New England Biolabs, Frankfurt, Germany). The ligated product was gel purified and subject to an 8 ml in vitro translation using rabbit reticulocyte lysate (Calbiochem-Novabiochem, Schwalbach, Germany) in the presence of ^{35}S-labeled methionine (Amersham Biosciences, Freiburg, Germany) for the first cycle. Volume was scaled down to 1 ml for all subsequent cycles. The resulting mRNA display molecules were purified on oligo-dT cellulose (Amersham Biosciences, Freiburg, Germany) and Ni-NTA agarose (Qiagen, Hilden, Germany). After reverse transcription, the complexity of the mRNA display peptides was calculated to be 1.2×10^{11} comparing the number of ^{35}S counts of the purified fusions to the counts of the total [^{35}S]methionine present in the translation mixture.

Selection

Human α-thrombin (Cell Systems, St. Katharinen, Germany) was coupled to CNBr-activated sepharose 4B (Amersham Biosciences, Freiburg, Germany) at 1 mg per 1 ml of swollen gel and the concentration on the matrix was calculated to be 27 μM. The purified mRNA display peptides were exchanged into selection buffer (140 mM NaCl, 4 mM $MgCl_2$, 5 mM KCl, 20 mM HEPES, 2 mM glutathione, 1 mM glutathione-disulfide, 0.1 mM EDTA, 0.25% Triton X-100 [pH 7.4]) over a preequilibrated NAP-10 column, which was blocked with 1 mg/ml salmon sperm DNA (Invitrogen, Karlsruhe, Germany) and 0.1 mg/ml BSA (Calbiochem, San Diego, CA). Incubation was then performed with 150 μl of thrombin matrix for 1 hr at 25°C with slight agitation. For cycles 2 onward, a NAP-5 column and only 100 μl of matrix were used. After appropriate washes with selection buffer (see Figure 1B), bound peptides were eluted with elution buffer (4 M urea, 0.5% SDS), desalted, precipitated, and subsequently PCR amplified.

Sequence Analysis

Amplified DNA from round 10 was digested with restriction enzymes KpnI and BamHI (New England Biolabs, Frankfurt, Germany) according to manufactures instructions and ligated into a complementary cut pGEM-4Z plasmid (Promega, Mannheim, Germany). *E. coli* DH10B cells were transformed and 72 colonies were randomly picked and grown up. In parallel dsDNA from the starting pool was treated the same way as described above and 36 sequences were analyzed (data not shown). The purified vectors were sequenced using Sequenase (Amersham Biosciences, Freiburg, Germany) and the dideoxy method in the presence of [α-^{35}S]dATP. DNA sequences for further analysis were commercially synthesized and PCR amplified using the primers mentioned in the library design section. For the truncated versions of the peptides lacking the his6 tag, a short 24-mer 3′-primer (5′-ACT GCC GCT CAT ACT GGA TCC GGA-3′) was used.

Binding Studies

To determine the relative binding of the selected peptides or their truncated versions, the dsDNA of the single clones or the pool was transcribed and the purified RNA directly subject to in vitro translation without ligation to the puromycin linker. According to a slightly modified protocol of Barrick et al. [34], a 20 μl aliquot of the crude lysate containing the free translated peptide was mixed with selection buffer and incubated with the thrombin matrix for 1 hr at 25°C with agitation. After draining for flow through, the sepharose was washed with selection buffer and then eluted with tricine-SDS loading buffer (50 mM Tris, 12% glycerol, 4% SDS, 0.1 M DTT [pH 6.8]). Identical aliquots of the elution fraction and the crude lysate were loaded onto a 12% tricine-SDS gel [50], run at 125 V, fixed, dried, and quantified using a phosphorimager (FUJI FLA3000). For competition studies, free human α-thrombin (10 to 5000 nM) was added to the samples, which were treated the same way as described above. K_i values were calculated as a function of percent binding inhibited versus the concentration of free thrombin.

K_d Determination and Epitope Mapping

Peptides of clones T10-11 and T10-39 lacking the his6 tag were synthesized by standard Fmoc-strategy and subsequently immobilized on a BIAcore CM5 chip using standard EDC/NHS coupling according to the manufactures instructions. Binding curves for thrombin (concentrations ranging from 1 to 2500 nM) were obtained and dissociation constants determined by steady-state affinity curve fits. To map the epitope which the peptides bind to, competition studies in solution were performed using hirudin (full-length, Roche, Mannheim, Germany), hirudin peptide (amino acids 54–65, nonsulfated and sulfated, Sigma, Taufkirchen, Germany), and antithrombin III (Roche, Mannheim, Germany). Thrombin was used at a constant concentration of 50 nM, and concentrations of the competitors were varied from 1 to 2500 nM. Further peptides representing either 7 or 8 amino acids of the minimal motifs, either C-terminally biotinylated or acetylated, were synthesized and used in the competition experiments.

SPOT Aanalysis

Peptide spots were synthesized by JERINI AG, Berlin, Germany. The cellulose membrane was blocked overnight with 10% blocking reagent (Roche, Mannheim, Germany), washed with T-TBS (50 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20 [pH 8.0]), and then incubated with human α-thrombin (10 μg/ml) for 75 min at 25°C followed by an incubation with a murine anti-thrombin antibody (Cell Systems, St. Katharinen, Germany) at 1 μg/ml for 75 min at 25°C. Finally, the membrane was incubated with a horseradish peroxidase coupled rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) at a dilution of 1:1000 for 75 min at 25°C. After 3 washes with T-TBS, the membrane was developed using ECL reagent (Amersham Biosciences) for 1.5 min and immediately exposed to a X-ray film for 5 s.

Clotting Studies

Thrombin clotting times were measured with a KC10 coagulometer (Amelung, Lemgo, Germany). One hundred microliters of frozen-thawed plasma (kind gift of Dr. B. Pötzsch) was preincubated at 37°C for 2 min. Then 100 μl of a solution containing 0.25 NIH units human α-thrombin, the inhibitor peptides, and $CaCl_2$ were added to give final concentrations of the peptides between 0.2 and 80 μM and 6.25 mM $CaCl_2$. All experiments were performed at least in triplicates.

Chromogenic Assays

The activity of thrombin was measured with the chromogenic substrate S-2238 (Haemochrom Diagnostica, Essen, Germany). One hundred fifty microliters of 50 mM Tris (pH 8.3) containing 0.5 NIH units human α-thrombin and increasing concentrations of the peptides were preincubated at 37°C for 5 min. Then 50 μl of a 2 mM aqueous solution of S-2238, prewarmed to 37°C for 5 min, was added and the measurement immediately started in a 96-well plate reader (Dynatech Laboratories, Chantilly, VA) at 405 nm. Relative initial reaction velocities, corrected by a control not containing a peptide, were plotted against the different peptide concentrations (ranging from 0.1 to 20 μM). Reactions were performed in duplicates.

Inhibition of protein C activation was determined using the chromogenic substrate S-2366, which is specific for activated protein C (aPC). A standard reaction in a microtiter plate contained 3.7 nM thrombin and 2 nM rabbit lung thrombomodulin in 50 mM Tris buffer (pH 7). This mix was preincubated at 37°C for 2 min. Then the peptides were added in concentrations ranging from 5 to 100 μM, followed by a 5 min incubation at 37°C. Final calcium concentration was adjusted to 2.5 mM and protein C was added to give 887 nM. After 15 min at 37°C, the chromogenic substrate was added and the measurement of the cleavage reaction immediately started in a plate reader at 405 nm. All measurements were performed in duplicates.

Model Generation

The model of the different inhibitors on thrombin was generated on a Silicon Graphics Octane Workstation (400 MHz R12k CPU, 1 GB RAM) running under SGI Irix 6.5 using the program package Sybyl 6.8 (Tripos Inc., St. Louis, MO). The available structural data of the complexes of thrombin with hirudin (4htc) [39], the DNA aptamer (1hap) [40, 41], and thrombomodulin (1dx5) [42] were superimposed and colored.

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