Chemistry & Biology
Article

An Exosite-Specific ssDNA Aptamer Inhibits the Anticoagulant Functions of Activated Protein C and Enhances Inhibition by Protein C Inhibitor

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DOI 10.1016/j.chembiol.2009.03.007

SUMMARY

Activated protein C (APC) is a serine protease with anticoagulant, anti-inflammatory, and cytoprotective properties. Using recombinant APC, we identified a class of single-stranded DNA aptamers (HS02) that selectively bind to APC with high affinity. Interaction of HS02 with APC modulates the protease activity in a way such that the anticoagulant functions of APC are inhibited and its reactivity toward the protein C inhibitor is augmented in a glysoaminoglycan-like fashion, whereas APC's antiapoptotic and cytoprotective functions remain unaffected. Based on these data, the binding site of HS02 was localized to the basic exosite of APC. These characteristics render the exosite-specific aptamers a promising tool for the development of APC assays and a potential therapeutic agent applicable for the selective control of APC's anticoagulant activity.

INTRODUCTION

The blood clotting process is a series of enzymatic reactions leading to the final formation of thrombin (Mann et al., 2003). To avoid overcoagulation with the risk of thrombus formation, this process is controlled by several anticoagulant mechanisms, such as the heparin-antithrombin pathway and the protein C- pathway (Dahlbäck and Villoutreix, 2005). The key enzyme of the protein C pathway is activated protein C (APC), a serine protease that is generated from its zymogen protein C (PC) (Esmon, 2003; Griffin et al., 2007). Formation of APC is a two-step process that occurs on the surface of endothelial cells in a thrombin-dependent manner (Griffin et al., 2007). It requires binding of PC to the endothelial cell PC receptor (EPCR), followed by proteolytic activation of PC through thrombin/thrombomodulin complexes (Esmon, 2003).

Once activated and dissociated from EPCR, APC regulates further thrombin formation by cleavage of its two main substrates, the activated cofactors V (FVa) and VIII (FVIIIa) (Dahlbäck and Villoutreix, 2005). In addition to its anticoagulant functions, APC shows cytoprotective effects, including anti-inflammatory and antiapoptotic activities, and protection of endothelial barrier function (Griffin et al., 2007). These direct effects of APC on cells require EPCR and the G protein-coupled receptor, protease activated receptor-1 (PAR-1) (Vu et al., 1991; Riewald et al., 2002).

The activity of the PC pathway is controlled by the rate of PC activation and APC inactivation. The major APC inactivator in human plasma is the protein C inhibitor (PCI), a member of the serpin superfamily (Huntington and Li, 2009). As found for other serpins, such as antithrombin and heparin cofactor II, addition of the glycosaminoglycan heparin augments the inactivation kinetics of APC by PCI (Aznar et al., 1996).

These multiple functions of APC require interactions with various plasmatic and cellular proteins. These molecular interactions are controlled and directed through amino acid sequences on APC's surface that are remote from the active site and termed exosites. Basic residues on three surface loops of APC, the 37-loop, the 60-loop, and the 70–80-loop, form a basic exosite that is involved in inactivation of factors Va and VIIIa (Dahlbäck and Villoutreix, 2005; Griffin et al., 2007). A second exosite formed by acidic residues of the 162 helix is located on the left side of the active site toward the back of the molecule and determines the specificity of APC in the interaction with PAR-1 (Figure 1A) (Yang et al., 2007).

The importance of the PC anticoagulant pathway is demonstrated by the life-long thrombotic tendency of patients showing inherited deficiencies of PC or other dysfunctions of the PC pathway as APC resistance (Dahlbäck and Villoutreix, 2005). Moreover, acquired PC deficiency contributes to the development of microvascular thrombosis in septicemia. Consequently, substitution with recombinant APC reduces mortality rates in severe sepsis (Bernard et al., 2001). A severe side effect of APC substitution, however, is major bleeding that occurs in 2% of patients (Levi, 2008). This severe side effect limits its clinical use. An APC-specific antidote would be helpful to manage APC-induced bleeding.

In addition, blockade of APC's anticoagulant activity might be an adjuvant approach for hemophilia treatment. Evidence that inhibition of APC might partially compensate for the absence of FVIII comes from in vitro studies with peptidomimetic or benzamidine-based APC inhibitors (Butenas et al., 2006; De Nanteuil

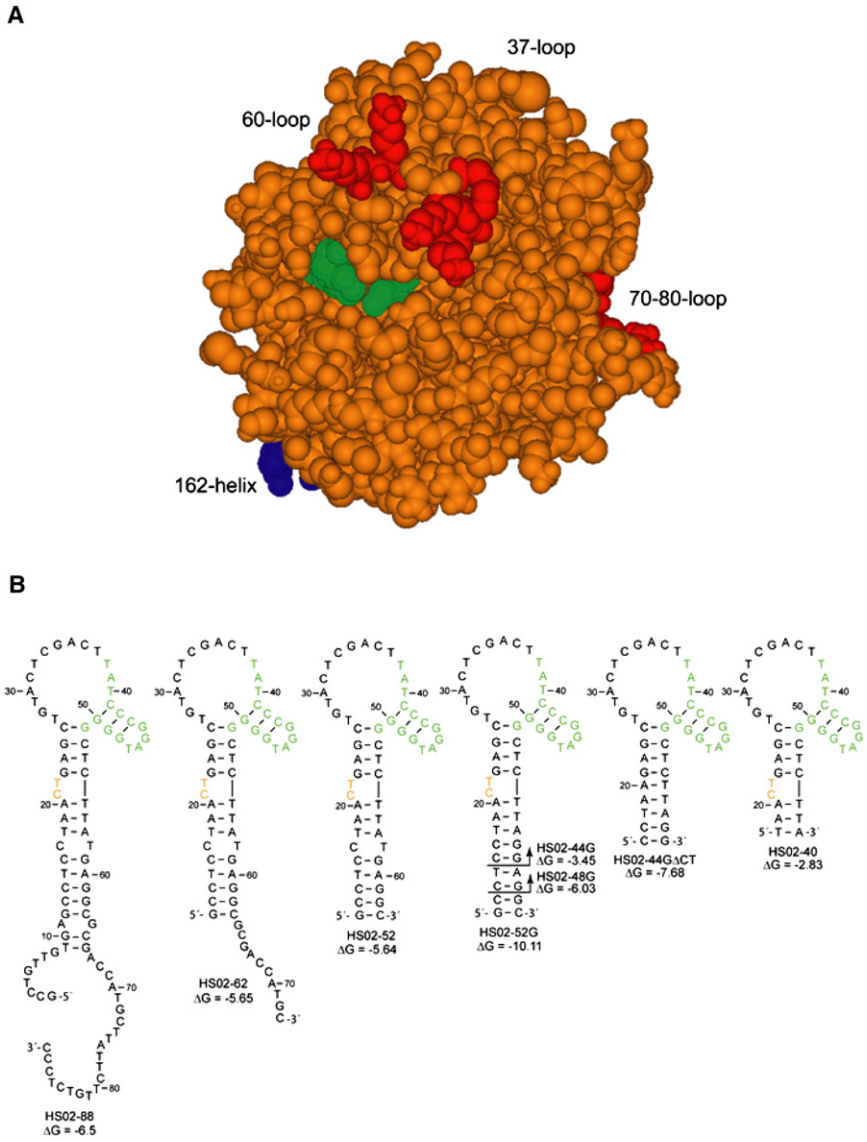

Figure 1. Structures of the Protease Domain of APC and In Vitro-Selected Aptamers

(A) Space-filling model of the crystal structure of APC based on the 1AUT structure deposited in the Protein Data Bank. The side chains of basic residues of the 39-loop, the 60-loop, and the 70–80-loop forming the basic exosite are shown in red, whereas acidic residues of the 162 helix are shown in blue. The catalytic triade is shown in green in the center of the molecule.

(B) Predicted secondary structures and stabilities (ΔG [kcal/mol]) of the HS02 aptamer variants. The base numbering consistently follows that of the full-length HS02-88 sequence.

et al., 2006). Both inhibitors, however, were not fully selective against thrombin, which might limit their therapeutic efficacy. This and the unknown toxicity of both substances emphasize the need for a nontoxic and highly specific APC inhibitor. In addition, a ligand that binds APC with high affinity but shows selectivity against the proenzyme PC can be used as a selective detector of APC and allows for the development of a test system for determining APC plasma levels.

Through their unique three-dimensional structure, aptamers are able to selectively interact with a variety of target molecules, ranging from proteins to peptides and small molecules. Their binding characteristics, their production properties, and their nontoxicity predestines them for being promising candidates for the development of clinically useable drugs (Famulok et al., 2007; Dollins et al., 2008). A 167 base RNA aptamer that binds to human APC with a K_D of ~100 nM has been reported (Gal et al., 1998). This aptamer inhibits the protease function of APC, including the cleavage reaction of a fluorogenic peptide substrate. The binding affinity of 100 nM is rather weak when compared to that of other aptamers that bind their corresponding targets with binding affinities in the low nanomolar or subnanomolar ranges (Gopinath et al., 2006; Müller et al., 2008a). Moreover, crossreactivity of this aptamer to PC has not been tested thus far (Gal et al., 1998). Another disadvantage of these molecules is their RNA structure, which makes them sensitive to enzymatic degradation in the plasma or whole-blood matrix.

To overcome these drawbacks, we selected single-stranded (ss) DNA aptamers targeting recombinant APC. Binding characteristics, the selectivity, and the binding region were evaluated. We demonstrate that the selected APC aptamers specifically target the heparin-binding site of the APC molecule, thereby inhibiting its anticoagulant functions and stimulating the inhibition of APC by PCI. In turn, the aptamers are rapidly released from the formed APC-PCI complexes. This represents a novel mechanism of an allosteric-like, aptamer-mediated protease inhibition. We conclude that the APC aptamers represent promising candidates for the development of clinically useable drugs specifically inhibiting APC activity in vivo and for the development of a rapid and selective test system that measures APC activity.

RESULTS

Aptamer Selection, Affinity, and Specificity

Prior to selection, APC was biotinylated and coupled to streptavidin-coated magnetic particles, which were then used directly during the in vitro selection (Müller et al., 2008b). As a nucleic acid library we utilized a ssDNA pool with a 49 nucleotides random region (Raddatz et al., 2008). After 12 selection cycles, the enriched library was analyzed for APC binding by employing filter retention analysis, and an increased affinity of the cycle 10 and cycle 12 ssDNA libraries compared to the starting library

Table 1. Binding Affinities of the HS02 Variants to APC and Related Serine Proteases and the Zymogen Protein C

	K_D, nM						
Aptamer	rAPC	pAPC	pPC	pFIIa	rFVIIa	pFIXa	pFXa
HS02-88	0.43 ± 0.08	0.47 ± 0.06	155 ± 19.9	N/A	N/A	N/A	N/A
HS02-62	0.56 ± 0.13	1.06 ± 0.23	177 ± 26.6	N/A	N/A	N/A	N/A
HS02-52	0.35 ± 0.09	0.97 ± 0.18	181 ± 18.3	N/A	N/A	N/A	N/A
HS02-52G	0.47 ± 0.11	0.74 ± 0.26	221 ± 46.8	N/A	N/A	N/A	N/A
HS02-48G	0.40 ± 0.13	0.85 ± 0.06	236 ± 106	N/A	N/A	N/A	N/A
HS02-44G	0.17 ± 0.06	0.76 ± 0.10	162 ± 60.9	N/A	N/A	N/A	N/A
HS02-44G-CT	8.75 ± 0.96	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HS02-40	4.57 ± 1.59	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

r, recombinant; p, plasma derived; N/A, not applicable (no binding observed up to 320 nM); n.d., not determined. Values are given as mean ± standard error.

was observed (data not shown) (Müller et al., 2008b). Cloning and sequencing individual members of the DNA library corresponding to the tenth selection cycle revealed a 14 base-spanning consensus motif, 5′-TATCMCGNATGGGS-3′ (see Figure S1 available online).

Among all analyzed ssDNA molecules, HS02-88 revealed the highest affinity (K_D value of 0.43 nM) and was chosen for further characterization. Several truncated variants of the aptamer HS02-88 were synthesized to determine the minimal sequence requirements for high-affinity rAPC binding. The sequences and in silico predicted secondary structures (Zuker, 2003) are shown in Figure 1B. The binding affinities of the aptamer variants were determined by filter retention analysis, and corresponding K_D values were calculated as described (for details, see Supplemental Data). As shown in Table 1, aptamer HS02-88 and the sequentially truncated variants HS02-62, HS02-52, HS02-52G, HS02-48G, and HS02-44G confirmed comparable binding affinities in the subnanomolar to low nanomolar range. A mean Hill-slope of 0.86 ± 0.14 as determined by a 4-parameter Hill logistic curve fit used for the calculation of K_D values indicated binding of the aptamers to APC in a 1:1 stoichiometry.

Markedly reduced affinities of the variants HS02-44G-ΔCT, lacking the CT bulge in the stem structure, and HS02-40, which comprises a truncated stem sequence, thus leading to a destabilized stem structure, were observed. These results suggest that an overall secondary structure that bears a stem with a CT bulge and an extended loop structure comprising the enriched 14 nucleotide consensus sequence contributes significantly to high-affinity APC binding. To determine the specificity of the HS02 aptamer variants, their binding activities to the proenzyme PC, to thrombin (FIIa), and to the activated factors VII (FVIIa), IX (FIXa), and X (FXa) were assessed by filter retention analysis. Up to a concentration of 320 nM, neither the aptamer HS02-88 nor its truncated forms showed binding to the factors IIa, VIIa, IXa, and Xa (Table 1). Binding to the proenzyme PC was detectable, but the binding affinities were 2–3 orders of magnitude lower compared to APC. Nearly identical binding affinities were obtained for plasma-derived APC and the recombinant version.

Influence of HS02 Aptamers on the Enzymatic Activities of APC

Having shown that the aptamers bind with high affinity and specificity to APC, we next analyzed their influence on the amidolytic activity of APC. This was investigated by using a fluorogenic peptide substrate assay. Hydrolysis rates at a final substrate concentration of 200 μM were partially inhibited in the presence of the full-length aptamer HS02-88 and its truncated forms, reaching a maximum reduction of 30.51% ± 2.56%, whereas the unselected D1 library showed no effect (Figure 2A). However, this effect diminished when higher concentrations of substrate were used, indicating that the aptamers alter the K_M for small peptide substrates (Figure S2).

The influence of the HS02 aptamer variants on the APC activity toward the biological substrates FVa and FVIIIa was measured by an FXa- and a thrombin-generation assay utilizing purified FVIIIa and FVa, respectively. Addition of the aptamers to the reaction mixtures resulted in a nearly complete inhibition of APC-mediated proteolysis of FVIIIa/FVa. As shown in Table S1, IC_{50} values for the inhibition of proteolysis of FVa and FVIIIa by APC were in the low nanomolar range and were comparable for all HS02 aptamers tested. No inhibitory effect was observed when using increasing concentrations of the unselected D1 library.

With respect to the kinetics of cofactor inactivation shown in Figure 2B, the aptamers significantly inhibited APC activity, as determined by the measurement of residual FVa/FVIIIa activity after incubation with rAPC in intervals up to 30 min. In the presence of 100 nM D1 library, the half-life times of FVa and FVIIIa in the described test systems were determined as 3.3 and 8.3 min, respectively. In the presence of 100 nM of the aptamer HS02-88 or its truncated variant HS02-44G, prothrombinase activity was nearly completely preserved, yielding a mean of 86.9% ± 0.98% of initial FVa cofactor activity (Figure 2B, upper panel). Similar results were obtained for the inhibition of APC activity toward FVIIIa (81.4% ± 1.84% of initial tenase activity) (Figure 2B, lower panel).

As determined by filter retention analysis, the HS02 aptamers bind with comparable affinities to both APC and APC/Protein S(PS) complexes (see Figure S3). To study the influence of PS on the inhibitory capacity of the HS02 aptamers, functional studies with purified PS have been performed. In the presence of PS, the half-life of FVa was reduced to 0.5 min, whereas in the presence of 100 nM HS02-88 or HS02-44G more than 50% of initial FVa cofactor activity was preserved after 30 min of incubation (Figure 2B, upper panel, inset). These results indicate that, in part, PS overcomes the inhibitory effects of the

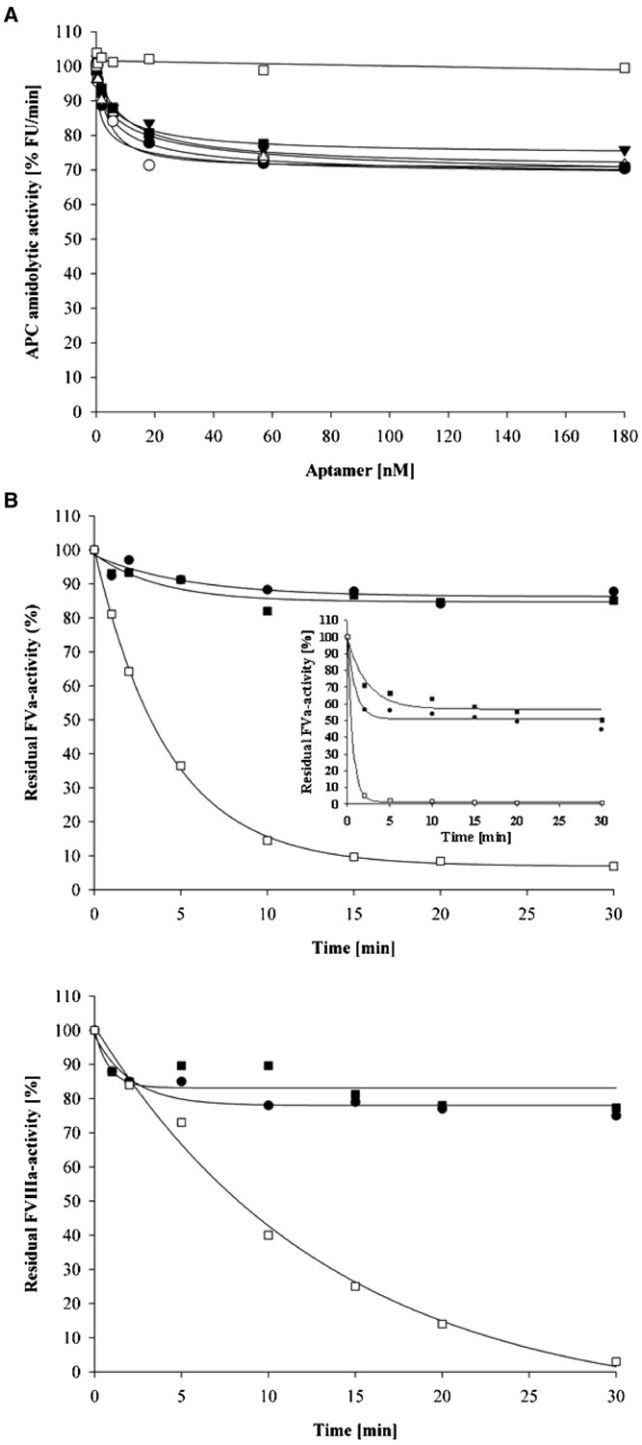

Figure 2. The Effect of the HS02 Aptamers on the Enzymatic Activity of APC

(A) The amidolytic activity of rAPC at a final concentration of 180 pM was measured in the presence of HS02-88 (solid circles), HS02-62 (open circles), HS02-52G (closed triangles), HS02-48G (open triangles), HS02-44G (closed squares), and the unselected D1 library (open squares). Results are shown as the mean of duplicates.

(B) The effect of the full-length HS02-88 aptamer (solid circles) and the truncated HS02-44G aptamer (solid squares) on APC-mediated inactivation of FVa and FVIIIa. Purified factors Va (upper panel) and VIIIa (lower panel) were incubated with APC in the presence of 100 nM of the aptamers, and the FVa/VIIIa activities were evaluated in the prothrombinase/tenase assay. The unselected D1 library was used as control (open squares). Factor Va inactivation patterns were also assessed in the presence of 150 nM Protein S (upper panel, inlet). All results are shown as means of duplicates.

aptamers bound to APC. Because PS has been shown to accelerate APC-mediated cleavage of FVa at Arg^{506} and Arg^{306}, no detailed information on cleavage site(s) preferences of APC/PS in the presence of the aptamers is available from the present data. However, the observed high residual activity of FVa in the absence of PS indicates that the aptamers interfere with APC-mediated cleavage at both Arg^{506} and Arg^{306} (Norstrøm et al., 2003).

Effect of the Aptamers on APC Anticoagulant Function in Plasma and Whole Blood

To test the influence of the HS02 aptamer variants on the anticoagulant function of APC in plasma and whole blood, an APC sensitivity test was used. Because previous studies revealed Ca^{2+}-dependent functional activity of the aptamers in plasma (see Figure S4), hirudin was chosen as the anticoagulant for these experiments. Reproducible baseline- and APC-mediated prolonged PT-clotting times were achieved at the applied concentrations of hirudin and dilution of the used TF reagent, allowing for reliable determination of concentration-dependent aptamer activity.

If APC was preincubated with the aptamers before being added to plasma, all tested aptamer variants efficiently inhibited APC anticoagulant activity with determined IC_{50} values in the low nanomolar range (mean IC_{50} value = 1.11 ± 0.23 nM).

As shown in Figure 3, however, addition of the aptamer HS02-88 or the truncated variants HS02-52G, HS02-48G, and HS02-44G to plasma or whole blood that already contained APC revealed different functional activities of the aptamers under this strategy. Variant HS02-52G was found to be the most effective variant, showing IC_{50} values of 1.6 ± 0.1 nM and 2.7 ± 0.2 nM in the plasma and whole-blood matrix, respectively. In contrast, the aptamer HS02-88 and its truncated variant HS02-44G were observed to be less effective. Because the stability of DNA aptamers in the plasma or whole-blood matrix was found to be high (Figure S5C) (Paul et al., 2008), the observed differences in functional activity might be traced to the structural stability and the target specificity of the aptamers in complex matrices.

Effect of the Aptamers on the APC-Mediated Inhibition of Staurosporine-Induced Apoptosis

The anticoagulant and antiapoptotic functions of APC are related to different subdomains of the protease. Therefore, we questioned whether the aptamers, which have already been shown to inhibit the anticoagulant function of APC, possess a domain that inhibits the anticoagulant functions of APC, but not its cytoprotective functions, and thus show inhibitory specificity. In this regard, the aptamer should not be able to interfere with the antiapoptotic function of APC. To test this hypothesis, cultured endothelial cells of passage 4 were incubated with the ATP analog staurosporine to induce apoptosis. Incubation with staurosporine resulted in an increased mean basal apoptotic rate

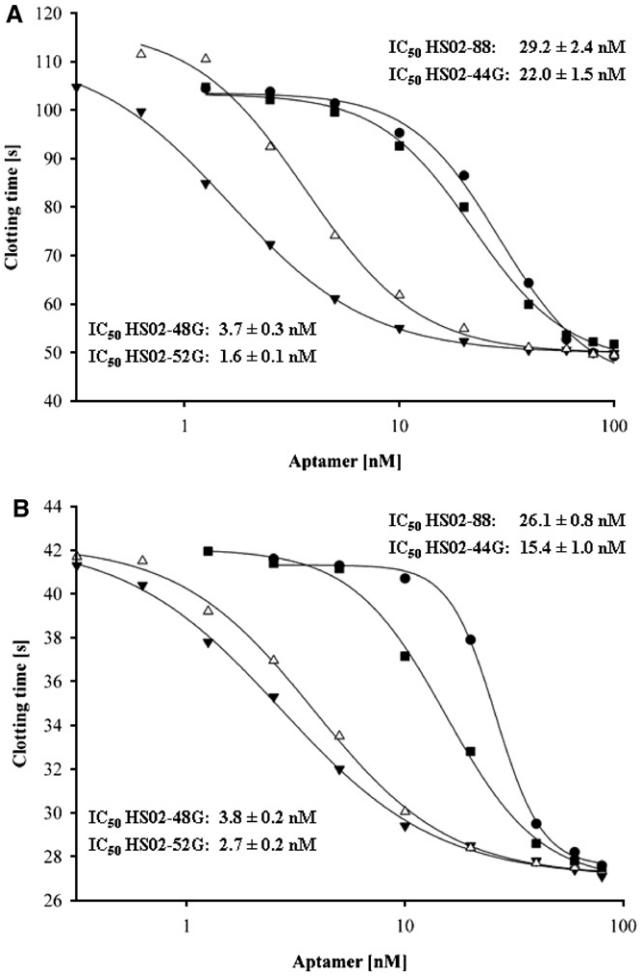

Figure 3. The Influence of HS02 Aptamers on APC Anticoagulant Function in Plasma and Whole Blood

(A and B) (A) Hirudin-anticoagulated plasma or (B) whole blood (B) was spiked with rAPC, and the full-length aptamer HS02-88 (solid circles) or the truncates HS02-52G (solid triangles), HS02-48G (open triangles), and HS02-44G (solid squares) were added at the indicated concentrations. Clotting was initiated by addition of tissue factor reagent. Results are shown as means of duplicates.

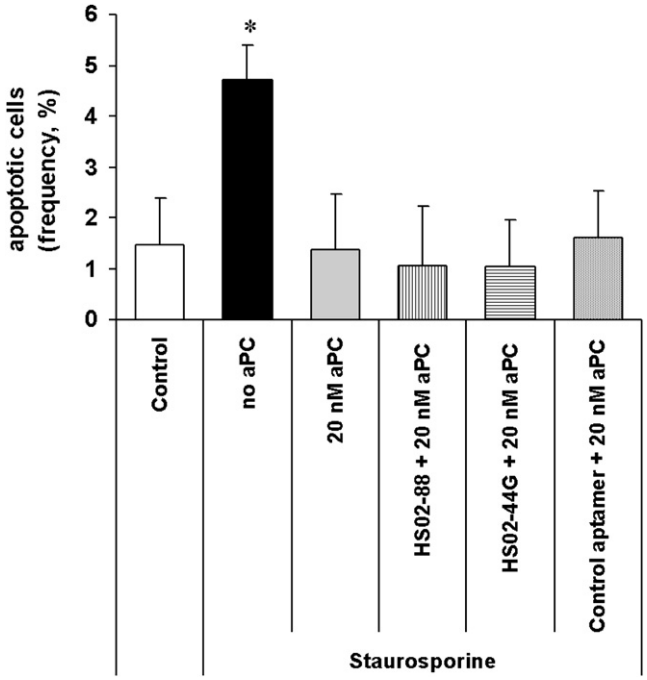

Figure 4. The Influence of HS02 Aptamers on APC Antiapoptotic Activity

Confluent monolayers of human umbilical vein endothelial cells of passage 4 were incubated overnight with 20 nM APC in the presence and absence of 100 nM HS02 aptamers. Apoptosis was induced by incubation with staurosporine at 2 μM for 4 hr. Then, the cells were fixed with 4% paraformaldehyde, and apoptotic nuclei were stained with the TUNEL reaction mixture. Results are shown as means with standard deviations of three independent experiments. *$p < 0.01$ (ANOVA).

(Figure 4). In contrast, endothelial cells that were preincubated with 20 nM APC over night (~12 hr) displayed a significant reduction of the apoptosis rate reaching the baseline level. As shown in Figure 4 and in accordance with our hypothesis, the antiapoptotic effect of APC was influenced neither by the aptamer HS02-88 nor by its truncated variant HS02-44G if added to the culture medium at a final concentration of 100 nM . Experiments with radioactively labeled HS02-88 molecules revealed that whereas the aptamers are stable in the medium itself (Figure S5B), the concentration of aptamers dropped to ~45 nM after 8 hr and to ~20 nM after overnight (~14 hr) incubation on human umbilical vein endothelial cells (HUVECs) under the given assay conditions (Figure S5A). Nevertheless, due to the high binding affinity of the HS02 aptamers to APC (K_D of ~0.5 nM), these concentrations should have been sufficient to complex all functional active APC molecules still remaining in the medium.

Influence of the Aptamers on APC Inactivation by the Protein C Inhibitor

Another ligand that interacts with APC is the PC inhibitor. To study the influence of the HS02 aptamers on the formation of APC-PCI complexes, immobilized APC was incubated with plasma as a source of PCI. The time-dependent formation of APC-PCI complexes was measured directly by using a PCI-specific antibody and indirectly through hydrolysis rates of a fluorogenic APC substrate. In the presence of 100 nM HS02 aptamers, APC inactivation rates were markedly enhanced (Figure 5A). The loss of APC activity correlated with the formation of APC-PCI complexes (Figure 5B). As shown in Table S2, there was a close correlation ($r = 0.95$) between the size of the HS02 aptamers and the apparent first-order constants (k_{app}) of APC inactivation, with the aptamer HS02-88 ($k_{app} = 0.1221 \pm 0.0158$) being the most effective variant compared to the control reaction without aptamer ($k_{app} = 0.0232 \pm 0.0045$).

To study whether the HS02 aptamers dissociate from APC after APC-PCI complexes have been formed, binding studies with biotinylated aptamers were performed. The results shown in Figure 5C demonstrate a decrease in the concentration of APC-bound aptamers, while the amount of APC-PCI complexes simultaneously increased (data not shown). In additional experiments, APC was bound to immobilized HS02 aptamers and subsequently incubated with plasma. Both PC activity and PC

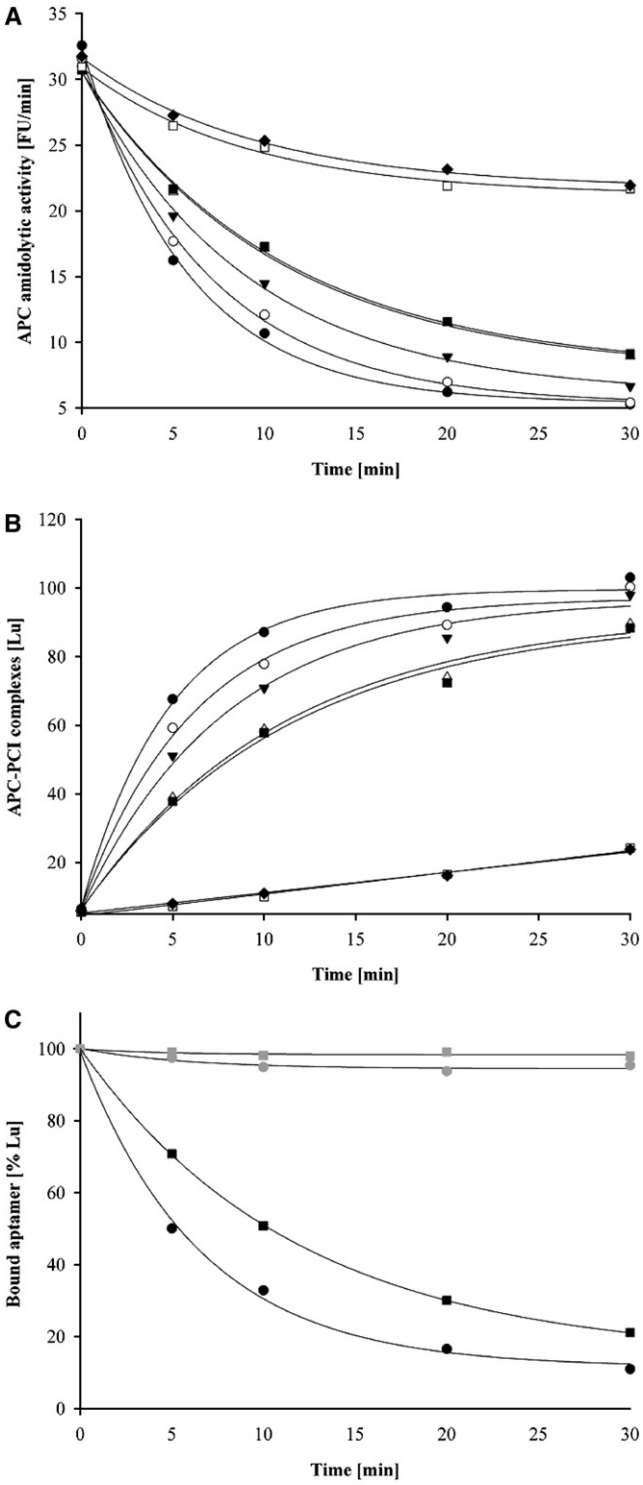

Figure 5. Inhibition of APC by PCI in the Presence of HS02 Aptamers

(A) Immobilized APC was incubated with plasma, and the time-dependent changes in APC activity were measured through hydrolysis rates of a fluorogenic APC substrate.

(B) Formed APC-PCI complexes were measured in an ELISA format utilizing a POD-labeled goat anti-human PCI antibody without aptamers (solid diamonds), 100 nM of unselected D1-library (open squares), HS02-88 (solid circles), HS02-62 (open circles), HS02-52G (solid triangles), HS02-48G (open triangles), and HS02-44G (solid squares).

(C) Release of HS02 aptamers upon APC-PCI complex formation. 3′-biotinylated variants of HS02-88 (circles) and HS02-44G (squares ■) were bound to immobilized APC prior to the addition of plasma or buffer. As determined by POD-labeled streptavidin, incubation with buffer (gray symbols) did not lead to a detectable release of aptamers over time. In contrast, addition of plasma initiated APC-PCI complex formation (data not shown) and a corresponding loss of streptavidin-mediated detection of aptamers (black symbols). Results are shown as means of duplicates.

antigen levels decreased over time, indicating that the formed APC-PCI complexes dissociate from the immobilized aptamers (Figure S6). Furthermore, no binding of plasma PCI could be detected in the wells.

Heparin Competition Experiments

Heparin has been shown to bind to the basic exosite of APC (Friedrich et al., 2001). To study whether the binding site of the HS02 aptamers is identical or overlaps with the binding region, competition experiments were performed. Increasing concentrations of unfractionated heparin (UFH) inhibited the binding of radioactively labeled aptamers to APC with a mean IC_{50} value of 6.73 ± 1.72 μM. The calculated mean Hill-slope of −1.07 ± 0.22 indicates that UFH and the aptamers competed for the same binding site on the APC molecules (Figure 6).

Influence of the Aptamers on PC Activation by Thrombin/Thrombomodulin

To study if the aptamers have an effect on PC activation by thrombin/thrombomodulin, an amidolytic APC-generation assay was used. As shown in Figure S7, aptamers HS02-88, HS02-52G, and HS02-44G interfered with APC generation in a size- and concentration-dependent manner. In accordance with the determined PC-binding affinities of the aptamers, however, this effect only became distinct at aptamer concentrations ≥ 200 nM.

DISCUSSION

We identified a novel, to our knowledge, class of high-affinity ssDNA ligands that selectively bind to human APC. Based on secondary structure analysis and the evaluation of truncated aptamers, a stem loop structure together with a CT bulge were found to represent a critical structural motif for high-affinity APC binding.

APC belongs to the group of vitamin K-dependent clotting factors that are characterized by the presence of a γ-carboxyglutamic acid domain at the amino end of the protein and show a high degree of structural homology (Stenflo, 1999). Filter retention analysis with activated forms of factors II, VII, IX, and X ruled out any significant binding of the APC aptamers to these structurally related proteases, thus emphasizing the high degree of specificity of the selected aptamer and its truncated variants.

The vitamin K-dependent clotting factors circulate in the blood stream in their zymogen variant until the coagulation pathway becomes activated. Aptamers to a variety of vitamin K-dependent clotting factors that bind their corresponding targets with K_D values ranging from ≈0.5 nM to 50 nM have been generated. Despite the fact that these aptamers were selected against the active enzyme, they bind the corresponding zymogens with

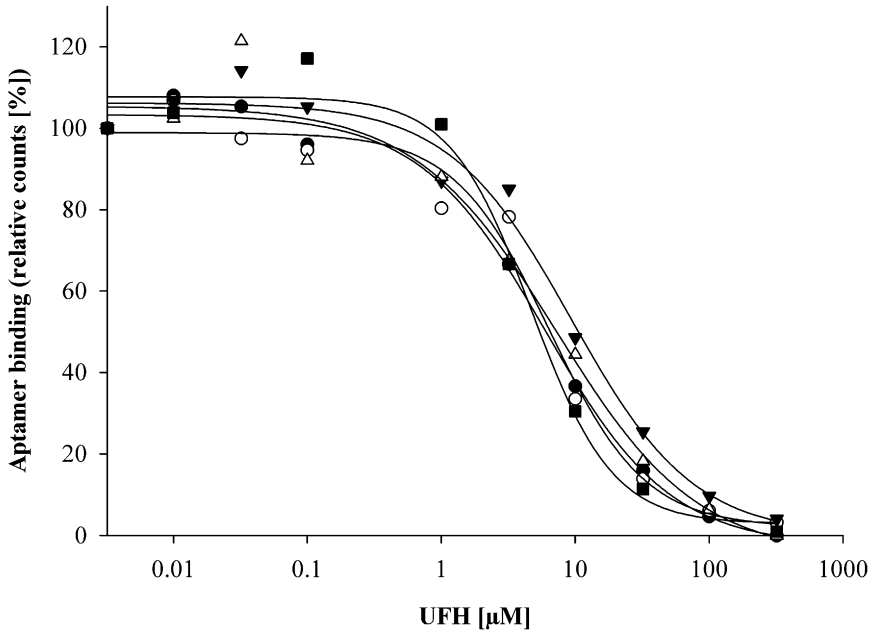

Figure 6. Heparin Competition Experiments
Radioactively labeled aptamers HS02-88 (closed circles), HS02-62 (open circles), HS02-52G (closed triangles), HS02-48G (open triangles), and HS02-44G (closed squares) were incubated with APC in the presence of increasing concentrations of UFH. Filter retention analysis indicated competitive inhibition of aptamer binding by UFH (mean Hill-slope = −1.07 ± 0.22; mean IC_{50} value = 6.73 ± 1.72 μM). Results are shown as means of duplicates.

comparable affinities (Kretz et al., 2006; Müller et al., 2008a; Rusconi et al., 2000; Gopinath et al., 2006). The binding affinities of the HS02 aptamer variants regarding PC were found to be ~200 times weaker than that observed for APC binding. This remarkable specificity renders the HS02 aptamer variants promising candidates for the development of rapid APC-specific assays, because the development of such an assay requires ligands with the ability to distinguish between PC and APC because the plasma level of PC is estimated to be 1000-fold greater than the concentration of APC. At present, only one monoclonal antibody (HAPC 1555) has been identified that shows a 10-fold higher affinity toward APC than to PC (Esmon et al., 2002).

To map the binding site of HS02 to APC, we performed a series of functional studies and competition experiments with known APC ligands. The amidolytic activity of APC is only partially influenced by HS02, indicating that the catalytic apparatus of APC is not directly blocked by HS02, but makes a binding region in close contact to the active site most likely. The active site cleft is flanked by two exosites that are involved in the interaction of APC with its macromolecular substrates. According to chymotrypsin numbering, one of these exosites, the basic exosite, comprises the 37-loop (Lys^{37}–Lys^{39}), the 60-loop (Lys^{62} and Lys^{63}), and the 70–80-loop (Arg^{74}, Arg^{75}, and Lys^{78}) of APC (Friedrich et al., 2001; Yang et al., 2002). The mutagenesis of basic residues of the 37-loop and the 70–80-loop has resulted in mutant proteases with markedly impaired anticoagulant activity, indicating that the basic exosite is involved in interaction of APC with factors Va and VIIIa (Friedrich et al., 2001; Yang et al., 2002; Gale et al., 2002; Manithody et al., 2003). The dose-dependent inhibition of APC-mediated cleavage of factors Va and VIIIa by HS02 suggests that the aptamers inhibit binding of FVa/FVIIIa to APC, most likely through blockade of the basic exosite. Another ligand that binds to APC through the basic exosite is heparin (Friedrich et al., 2001). Heparin competitively inhibits binding of HS02 aptamers to APC. Thus, these data provide further evidence that the basic exosite is indeed the binding site of the HS02 aptamer variants.

Heparin binding to APC has been shown to accelerate the PCI-mediated inhibition of APC in a molecular weight-dependent manner (Aznar et al., 1996). It is anticipated that heparin induces the formation of a ternary complex between heparin, APC, and PCI, and that inhibition of APC by PCI works by a template mechanism wherein APC and PCI bind to the same heparin molecule (Li and Huntington, 2008). To study whether aptamer binding interferes with PCI-mediated inactivation of APC, we performed a series of APC inhibition experiments. The HS02 aptamers accelerate the inactivation kinetics of APC by PCI in a concentration- and size-dependent manner. However, after APC-PCI complex formation, the aptamers were shown to be rapidly released, revealing a novel aptamer-based inhibitory mechanism. These observations might indicate that the aptamer-binding epitope of APC is not directly involved in the complex formation between APC and PCI, but that the conformational changes induced by APC-PCI complex formation alters this epitope in a way that consequently leads to the release of the aptamer. In contrast to heparin, the aptamers did not show any binding to PCI or APC-PCI complexes, making it most unlikely that they are directly involved in the formation of a ternary complex through a template mechanism. Thus, allosteric changes within the APC molecule upon aptamer binding might account for the observed effects. Interestingly, a comparable mechanism has been reported previously for an RNA aptamer that binds the anion-binding exosite 2 of thrombin (Jeter et al., 2004). Taken together, these results make the HS02 aptamers interesting molecular tools for studies on the interaction between APC and PCI.

A second exosite is located within the APC molecule opposite to the basic exosite on the left of the active site toward the back of the molecule and is formed by acidic residues of the 162 helix. This acidic exosite determines the specificity of the protease interaction with PAR-1 and thereby determines its cytoprotective properties (Yang et al., 2007). To test, if the cytoprotective functions of APC are influenced by aptamer binding, we performed a staurosporine-induced apoptosis assay with HUVECs. This is an established model system by which to study antiapoptotic effects of APC (Isermann et al., 2007; Bae et al., 2007). Whereas APC significantly reduced the staurosporine-induced apoptosis rates, none of the HS02 aptamers inhibit this cytoprotective effect of APC. These results support previous findings obtained

with APC mutants showing that the basic exosite is not involved in the cytoprotective functions of APC and gave additional evidence that the binding site of the HS02 aptamers is located in the basic exosite of APC (Bae et al., 2007; Mosnier et al., 2004).

Although effective in the treatment of severe sepsis, administration of rAPC is complicated by the occurrence of major and life-threatening bleeding complications in 2% of patients (Levi, 2008; Vincent et al., 2005). These bleeding complications are induced by the anticoagulant functions of APC, whereas several in vitro and in vivo data indicate that the therapeutic efficacy of APC in severe sepsis is mainly due to APC's cytoprotective effects (Toltl et al., 2008). It is therefore tempting to speculate that the ability to selectively inhibit APC's anticoagulant activity makes the aptamers promising therapeutic agents to be used for the treatment of APC-induced bleeding complications.

Other interactions of APC with macromolecular substrates involve binding of APC to its cofactor PS. Using the newly characterized aptamers, we were able to show that the basic exosite is not involved in binding of APC to PS. These data extend and confirm recently published data showing that PS primarily binds to the GLA domain region of APC (Harmon et al., 2008).

In summary, we have identified and characterized ssDNA aptamers that bind with high affinity to the basic exosite of APC. Through blockade of this exosite, the APC aptamers act as selective and potent inhibitors of APC's anticoagulant functions. Simultaneously, binding of HS02 to APC enhanced inactivation rates of the protease by the serpin PCI. The antiapoptotic effect of APC is not inhibited by the APC aptamers, indicating that the cytoprotective effects of APC remain intact after aptamer binding. The aptamer represents an excellent starting point for the development of selective and sensitive diagnostic formats and APC-modulating drugs.

SIGNIFICANCE

APC controls the clotting process by downregulation of thrombin formation and possesses anti-inflammatory and cytoprotective functions. Given these important physiological functions of APC, determination of plasma levels of this protease are of clinical interest in the characterization of a hypercoagulable state and/or an inflammatory state. Specific detection and quantification of APC are hampered by the fact that a highly selective ligand that discriminates between APC and its precursor PC is not available. To circumvent this limitation, we used the aptamer technology to select a class of high-affinity (K_D of $\approx$0.5 nM) ssDNA APC aptamers, designated HS02. Binding to the zymogen PC was $\sim$200-fold weaker, making the HS02 aptamers promising ligands for the development of APC-specific assays. APC-related bleeding complications are a relevant problem in patients receiving recombinant APC for the treatment of severe sepsis. The HS02 aptamers inhibit the anticoagulant functions of APC by binding to the basic exosite of APC, but not the antiapoptotic and cytoprotective functions of the enzyme. The ability to selectively inhibit APC's anticoagulant functions makes HS02 a potential therapeutic agent for the treatment of APC-related bleeding. Finally, the exosite specificity of the APC aptamer makes it an interesting molecular tool by which to probe APC's interactions with several macromolecular substrates and cellular receptors.

EXPERIMENTAL PROCEDURES

Proteins, Reagents, and Oligonucleotide Library

A recombinant version of human APC (rAPC, Xigris) was purchased from Eli Lilly (Indianapolis, USA), and recombinant human Factor VIIa (NovoSeven) was purchased from Novo Nordisk (Bagsværd, Denmark). The plasma-derived human PC concentrate (Ceprotin) and recombinant FVIII (Recombinate) were purchased from Baxter (Unterschleißheim, Germany). Ceprotin was activated to APC by using human α-thrombin (for details, see Supplemental Data). All other coagulation enzymes were purchased from CellSystems (St. Katharinen, Germany) unless otherwise stated. Argatroban was obtained from Mitsubishi Pharma (Düsseldorf, Germany). Unfractionated heparin (UFH, Liquemin), the TUNEL assay kit, POD-labeled streptavidin, and the BM chemiluminescence substrate were obtained from Roche (Mannheim, Germany). Endothelial cell basal medium (ECBM) and FCS were purchased from Promocell (Heidelberg, Germany). The fluorogenic substrates PCa 3342 (Pyr-CHG-Arg-AMC) and PCa 5791 (Pyr-Pro-Arg-AMC) were purchased from Loxo (Dossenheim, Germany), and the rabbit anti-human PC antibodies and POD-labeled goat anti-rabbit antibodies were obtained from Dako (Glostrup, Denmark). The POD-conjugated goat anti-human PCI antibody was purchased from Haemochrom Diagnostica (Essen, Germany). Rabbit thrombomodulin was purchased from American Diagnostica (Pfungstadt, Germany). Streptavidin was purchased from Applichem (Darmstadt, Germany). The fluorogenic substrates Z-Gly-Gly-Arg-AMC and Boc-Ile-Glu-Gly-Arg-AMC were purchased from Bachem (Weil am Rhein, Germany). The EZ-link biotin hydrazide (NHS-Biotin) was purchased from Perbio Science (Bonn, Germany). The tissue factor reagent Innovin and the aPTT reagent Actin FS and soybean phospholipids were purchased from Siemens Healthcare Diagnostics (former Dade Behring, Marburg, Germany). For the preparation of a stock solution, dried soybean phospholipids were dispersed in water by using a water bath sonicator to obtain a final concentration of 1 μg/μl (1.3 mM). Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin) were purchased from Invitrogen (Karlsruhe, Germany).

An HPLC-purified ssDNA library (D1) that contained a randomized sequence of 49 nucleotides flanked by 2 primer hybridization sites (5′-GCCTG TTGTGAGCCTCCTAAC-N_{49}-CATGCTTATTCTTGTCTCCC-3′) was obtained from Metabion (Martinsried, Germany) and used as the primary library for the SELEX procedure. The corresponding amplification primers were purchased from Operon (Cologne, Germany).

SELEX

To enable the removal of unbound ssDNA molecules during the SELEX process, rAPC was coupled to magnetic beads for separation. First, 1.82 μmol (100 μg) reconstituted rAPC was incubated with 5.46 μmol NHS-Biotin in a total volume of 100 μl for 30 min on ice, followed by a 10 min incubation at room temperature. To remove unbound NHS-Biotin, the reaction mixture was applied to a Micro Bio Spin 6 chromatography column according to the manufacturer's instructions (Biorad, Munich, Germany). Purified rAPC-Biotin (100 μg) was added to 500 μl binding buffer (1× PBS [pH 7.4], 1 mg/ml BSA) containing 5 mg streptavidin-coated magnetic beads and incubated at room temperature for 30 min under constant agitation. After incubation, primed beads (rAPC beads) were washed and stored in 1 ml binding buffer at 4°C until used. The SELEX procedure was started by incubation of 500 pmol D1 library with 400 μg rAPC beads in a total volume of 160 μl selection buffer (PBS [pH 7.4], 1 mM each $CaCl_2$ and $MgCl_2$, 1 mg/ml BSA) for 30 min at 37°C. After washing with selection buffer, rAPC-bound aptamers were eluted by incubation in 55 μl water at 80°C for 3 min and reamplified with primers 5′-GCCTGTTGTGAGCCTCCTAAC-3′ (D1F, forward) and 5′-GGG AGACAAGAATAAGCATG-3′ (D1RB, reverse). Reverse primers were biotinylated at the 5′ end to enable the removal of reverse complement sequences by using streptavidin-coated magnetic beads as previously described (Raddatz et al., 2008). After denaturation of the double-stranded PCR products, 50 pmol isolated sense strands was introduced to the subsequent rounds of SELEX. From the third round, selected aptamers were preincubated with 400 μg nonloaded streptavidin-coated magnetic beads in order to eliminate potentially present sequences binding to streptavidin or bead structures. Twelve iterations of SELEX were performed. Due to the increasing amount of target-binding aptamers, the number of PCR cycles was reduced from

initially 18 (rounds 1–4) to 9–12 (rounds 5–12). To gradually increase the stringency of the selection process, the number of performed washing cycles was doubled after each round, starting from 1 to a maximum of 8 (rounds 4–12). The selection process was monitored by dHPLC analysis as previously described (Müller et al., 2008b). Detailed information on cloning of selected aptamers, sequence analysis, and the synthesis of candidate aptamers and controls is given in Supplemental Data.

Catalytic Activity of APC against Small Substrates

To assess the influence of aptamer binding on the amidolytic activity of APC, 50 μl 360 pM (20 ng/ml) rAPC in selection buffer containing different concentrations of aptamers or controls (0–360 nM) was placed into wells of white F8 Fluoronunc modules (Thermo Fisher Scientific, Nunc, Wiesbaden, Germany). Subsequently, 50 μl 400 μM Pyr-CHG-Arg-AMC (Pefa 3342) was added and substrate hydrolysis was monitored at 30°C by using an automated plate fluorescence/luminescence reader (Ascent Fluoroscan, Thermo Fisher Scientific, Dreieich, Germany). Alternatively, APC-amidolytic activity was determined in the absence or presence of 100 nM aptamers HS02-88 or HS02-44G and 0.01–2.5 mM Pefa 3342 (final concentrations).

Anticoagulant Activity of APC

The influence of aptamer binding on the anticoagulant activity of APC was measured by using a prothrombin-time (PT)-based one-stage clotting assay. Two different strategies were applied. First, 3.6 nM (200 ng/ml) APC was preincubated with 0–250 nM of the aptamers in TBS buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA). Subsequently, 20 μl of this solution was added to 30 μl hirudin-anticoagulated plasma (10 μg/ml hirudin) and incubated for 2 min at 37°C. To start the clotting reaction, 60 μl tissue factor reagent (Innovin, 1:2 diluted in TBS) was added.

Second, 30 μl hirudin-anticoagulated whole blood (5 μg/ml hirudin) or the corresponding plasma was spiked with 10 μl rAPC in TBS buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA) to achieve final concentrations of 2.6 nM (140 ng/ml) or 550 pM (30 ng/ml), respectively. Subsequently, 10 μl aptamers (0–500 nM) in TBS buffer was added. After incubation for 3 min at 37°C, coagulation was initiated by the addition of 60 μl tissue factor reagent (Innovin, 1:2 diluted in TBS). All measurements were automatically performed by using the coagulation analyzer AMAX CS 190 (Amelung, Lemgo, Germany).

Monitoring of FVa/FVIIIa Inactivation by APC

APC-mediated inactivation of FVIIIa and FVa was monitored in a purified system by using an FXa-generation assay and a thrombin-generation assay, respectively. Both assays were performed in 96-well microtiter plates.

Activated FVIII used for the FXa-generation assay was prepared by incubation of 1 U recombinant human FVIII (Recombinate) with 0.025 U human α-thrombin in PBS buffer ([pH 7.4] 1 mg/ml BSA) in a total volume of 100 μl. After 2 min of incubation at room temperature, 5 μl 1.4 nM hirudin was added to terminate thrombin activity. Activated FVIII at a concentration of 0.16 U/ml was incubated with 1.8 nM rAPC in assay buffer (20 mM Tris-HCl [pH 7.6], 137 nM NaCl, 10 μg/ml phospholipids, 5 mM $CaCl_2$, 1 mg/ml BSA) in the absence or presence of aptamers or controls (0.06–180 nM). After incubation for up to 30 min, 25 μl of the mixtures was transferred to the wells of black F16 Fluoronunc modules (Thermo Fisher Scientific, Nunc) containing 3 nM human FIXa and 333 μM Boc-Ile-Glu-Gly-Arg-AMC in a total volume of 75 μl assay buffer. Subsequently, 50 μl 25 nM human FX in assay buffer was added to the wells and the kinetics of FXa-mediated substrate hydrolysis monitored at 30°C by using an automated plate fluorescence reader (FLx-800, Bio-Tek, Bad Friedrichshall, Germany).

For determination of FVa inactivation, 625 pM FVa was incubated at room temperature with 18 pM rAPC in the absence or presence of 150 nM PS in assay buffer containing different concentrations of aptamers or controls (0–180 nM). After incubation for up to 30 min, 25 μl of the mixtures was transferred to the wells of white F8 Fluoronunc modules (Thermo Fisher Scientific, Nunc) containing 42 pM human FXa and 667 μM fluorogenic peptide substrate Z-Gly-Gly-Arg-AMC in a total volume of 75 μl in assay buffer. Subsequently, 50 μl 25 nM human prothrombin in assay buffer was added, and the kinetics of thrombin-mediated substrate hydrolysis was monitored by using the Ascent reader.

Residual cofactor activities were calculated according to a parallel-processed dilution series of FVa or FVIIIa in the absence of APC and aptamers during the first incubation step.

Staurosporine-Apoptosis Assay

Human umbilical vein endothelial cells (HUVECs) were used for this experiment. Aptamers (HS02-88, HS02-44G and the scrambled control sequence) were preincubated with APC in selection buffer for 5 min at 37°C. HUVECs of passage 4 grown to confluency were pretreated with 20 nM APC alone as well as with 20 nM APC along with 100 nM aptamers in ECBM (0.5% FCS) overnight (~12 hr). Then, the cells were incubated with 2 μM staurosporine (Sigma-Aldrich, Germany) for 4 hr to induce apoptosis. Cells were fixed with 4% paraformaldehyde, and apoptotic nuclei were stained by using the TUNEL assay kit.

Stability of the HS02 Aptamers on HUVECs and in Whole Blood

To assess the extracellular half-life of the aptamers in the presence of HUVECs as well as their stability in the used ECBM, 1% of radioactively labeled HS02-88 molecules were introduced together with unlabelled molecules at a final concentration of 100 nM to the culture medium and either incubated overnight on confluent HUVECs or in medium alone. For determination of aptamer stability in whole blood, the same mixture of aptamers was added to hirudin-anticoagulated whole blood (100 nM final concentration) and incubated at 37°C for up to 2 hr. Generally, samples were taken at different time points, and aptamers were isolated by phenol-chloroform extraction from medium or plasma, assessed for quantity and structural integrity on 8% PAGE gels with the radioactivity exposed on storage phosphor screens, and subsequently analyzed as described above.

Monitoring of APC Inactivation by PCI and Release of Aptamers from APC-PCI Complexes

Inactivation rates of immobilized APC by PCI were studied in the absence and presence of aptamers by using combined immunological and functional assays. Human plasma was used as a PCI source. The assays were performed in the microtiter plate format (for details, see Supplemental Data).

APC-Generation Assay

Formation of APC by thrombin/thrombomodulin complexes was monitored by using a fluorogenic assay as previously described (Müller et al., 2008a), with slight modifications. A detailed description of the performed assay is given in the Supplemental Data.

Data Analysis

All experiments were performed in duplicate, and data are shown as mean values. Sequence alignments were done by using the ClustalW tool available on the EMBI-EBI homepage (http://www.ebi.ac.uk/clustalW). Secondary structure analysis of aptamer sequences was done by using the Mfold web server for nucleic acid folding available at http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi (Zuker, 2003). Dissociation constants (K_D) and IC_{50} values were calculated by a 4-parameter Hill logistic curve fit. Inhibition, loss of binding, and complex formation data were interpolated by a 3-parameter exponential curve fit. Apparent first-order constants (k_{app}) of APC inactivation were determined by an exponential curve fit according to $x = x_0 * e^{-kapp * t}$, where x is the residual amidolytic rAPC activity at time point t (0–10 min) and x_0 is the fitted amidolytic APC activity in the buffer control. All calculations were done by using SigmaPlot 9.0 or Excel 2003 software.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00110-0.

ACKNOWLEDGMENTS

This work is part of the thesis of C.D., M.M., and M.F. G.M. acknowledges financial support from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The authors would like to thank Nicole Kuhn, Volkmar Fieberg, Susan Page, Judith Junen, and Alexandra Schmidt for expert technical assistance.

Received: October 13, 2008
Revised: February 25, 2009
Accepted: March 5, 2009
Published: April 23, 2009

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