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Analysis of the substrate specificity of Factor VII activating protease (FSAP) and design of specific and sensitive peptide substrates.

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ABSTRACT

Factor VII (FVII) activating protease (FSAP) is a circulating serine protease that is likely to be involved in a number of disease conditions such as stroke, atherosclerosis, liver fibrosis, thrombosis and cancer. To date, no systematic information is available about the substrate specificity of FSAP. Applying phage display and positional scanning substrate combinatorial library (PS-SCL) approaches we have characterized the specificity of FSAP towards small peptides. Results were evaluated in the context of known protein substrates as well as molecular modelling of the peptides in the active site of FSAP. The representative FSAP-cleaved sequence obtained from the phage display method was Val-Leu-Lys-Arg-Ser (P4-P1'). The sequence **X-Lys/Arg-Nle-Lys/Arg** (P4-P1) was derived from the PS-SCL method. These results show a predilection for cleavage at a cluster of basic amino acids on the nonprime side. Quenched fluorescent substrate (Ala-Lys-Nle-Arg-AMC) (amino methyl coumarin) **and (Ala-Leu-Lys-Arg-AMC) were** had a higher selectivity for FSAP compared to other proteases from the hemostasis system. **These substrates** could be used to measure FSAP activity in a complex biological system such as plasma. In histone-treated plasma there was a specific activation of **pro**-FSAP as validated by the use of an **FSAP** inhibitory antibody, **corn trypsin inhibitor to inhibit Factor XIIa and hirudin to inhibit thrombin**, which may account for some of the hemostasis-related effects of histones. These results will aid the development of further selective FSAP activity probes as well as specific inhibitors that will help to increase the understanding of the functions of FSAP *in vivo*.

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INTRODUCTION

FSAP is a circulating serine protease that is secreted by the liver as an inactive zymogen (1). Zymogen activation is mediated *in vivo* and in plasma by factors released from damaged cells such as histones and nucleosomes (2). Once activated, FSAP can be rapidly inhibited by serine protease inhibitors such as α1-proteinase inhibitor, α2-plasmin inhibitor, antithrombin, C1 inhibitor (3-6), as well as plasminogen activator inhibitor-1 (PAI-1) (7) and protease nexin-1 (8).

About 5% of the Caucasian population are carriers of a single nucleotide polymorphism (SNP) in the FSAP gene, G534E (Marburg I, MI), that results in an exchange of a single amino acid in the protease domain (9). MI-FSAP has about a 5-fold lower proteolytic activity than its WT counterpart towards various proteolytic substrates such as pro-urokinase, factor VII, tissue factor pathway inhibitor and high MW kininogen (10). MI SNP was found to be strongly linked to late complications of carotid stenosis (11), liver fibrosis (12), stroke (13), venous thromboembolism (14-16) as well as thyroid cancer (17, 18); however, it should be noted that some of these findings have been controversial. When tested in disease model systems, $FSAP^{-/-}$ mice have more severe liver fibrosis (19), stroke (20), neointima formation (21) but are protected against thrombosis (22). Thus the human genetic data and those from knock-out mice indicate that FSAP may play an important role in many patho-physiological conditions and thus reliable methods are needed to specifically and directly measure FSAP activity in the plasma as well as to localize active FSAP in tissue. Furthermore, to elucidate the molecular mechanisms involved in these actions of FSAP, as well as to further develop therapeutic concepts, it is necessary to understand its substrate specificity in more detail.

We have used two different approaches that, theoretically, allow a comprehensive screen of all possible peptide substrate sequences. Substrate specificity data from the phage display and positional scanning substrate combinatorial library (PS-SCL) methods **were**

further consolidated with the analysis of known FSAP substrates as well as modelling of the FSAP protease domain. FSAP has a strong preference for a cluster of basic amino acids on the non-prime side of the cleaved peptide bond. Based on these data, quenched fluorescent peptide substrates with a higher selectivity for FSAP over other hemostasis factors were identified. These novel substrates could be used to measure the specific activation of FSAP by histones in a complex biological fluid such as plasma.

MATERIAL AND METHODS

Phage display: The ANL16 phage library was kindly provided by Dr. B. Kay (University of Illinois, Chicago, USA) (24). The library consists of randomized 7 AA sequences cloned between a Bir-A sequence which is recognized by Bir-A transferase to initiate biotinylation at the N-terminus and the pIII gene which encodes the minor coat protein pIII of the M-13 filamentous phage. Initially, phages were grown and biotinylated *in vivo* by infecting F-positive *E. Coli* AVB100FmkII (Str/Tetr) (Avidity LLC, Aurora, CO, USA) which harbors an L-arabinose inducible bir-A cassette in its genome as described elsewhere (23). Phages in the bacterial culture were purified and concentrated by PEG/NaCl as described before (23). 100 µl Dynabeads® MyOne™ Streptavidin T1 (ThermoFisher Scientific, Oslo, Norway) were blocked with TBST/BSA (TBS supplemented with 0.01% v/v Tween 20 and 3% w/v BSA) for 1 h at 22°C. ANL16 substrate phage library was blocked with TBST/BSA. $4.3X10^{10}$ phages were incubated with beads for 1h at RT and washed extensively with TBST and incubated with **150 nM (10 µg/ml)** FSAP in reaction buffer TBST80/$CaCl_2$ (1 mM), for 1 h at 37°C. Supernatant with phages released by FSAP were collected and F-positive AVB100FmkII bacteria were infected. Phages were grown and biotinylated in bacteria culture for 18 h at 37°C. After PEG/NaCl purification, phages were used for the next round of bio-panning. After three rounds of bio-panning, 500 individual phage clones were selected by plaque assay and amplified as described before (23).

Phage substrate enzyme-linked immunosorbent assays **(ELISA)**: **Phage ELISA was performed as described before (24).** MaxiSorpTM microtiter plate wells (Nunc, Roskilde, Denmark) were coated with 50 µl of 2 µg/ml neutravidin (ThermoFisher Scientific, Oslo, Norway) in PBS at 4°C for 18 h. The wells were blocked with 100 µl TBST/BSA for 1 h at 22°C. Amplified single phage clone supernatants were added to wells and incubated for 1 h at RT. After washing the wells, **30 nM** FSAP in reaction buffer TBST/ $CaCl_2$ (2 mM) or an equivalent volume of buffer in which the FSAP was stored (0.2 M Arginine, 0.2 M Lysine, 5 mM Na Citrate, pH 4.5) was added to the wells. The top of the micro plate was sealed with MicroAmp® Optical Adhesive Film (ThermoFisher Scientific, Oslo, Norway) and incubated at 37°C for 18 h. The micro-plate was washed and the phages detected with anti-M13-HRP (1:5,000 dilution in TBST/ BSA) (GE Healthcare, Uppsala, Sweden). The signal was developed with 1-Step Ultra TMB ELISA Substrate (ThermoFisher Scientific, Oslo, Norway), and the reaction was stopped with 2 M sulfuric acid and absorbance measured at 450 nm with a Synergy H1 Multi-Mode Reader (BioTek, Winooski, VT, USA). The relative **phage release for** each phage clone was **determined as** the ratio of signals from reactions with FSAP compared to control buffer. Primers pIII FRWD: 5'-TTT TAG GAG ATT TTC AAC GTG-3' and -96 phage: 5'-CCC TCA TAG TTA GCG TAA CG-3' were used in PCR reactions with phage template from an aliquot of phage culture to amplify the M13 phage pIII gene region with the randomized peptide library. Analysis of peptide sequences was performed using online software JABALS 2.1, CLUSTAL format alignment by MAFFT FFT-NS-2 (v7.245)/ MafftWS version 6.8.57, colored by ClustalX format (25) and represented by iceLogo (26).

Positional scanning synthetic combinatorial library (PS-SCL): Briefly, a tetrameric complete diverse PS-SCL was used essentially as described before (27). The final substrate concentration in the library assays was 250 µM and the final FSAP concentration was **30 nM**

in Tris (50 mM) (pH 8.8), NaCl (100 mM), $CaCl_2$ (1 mM), Tween-20 (0.01% v/v), DMSO (1% v/v). All library assays were performed in 96-well plates (Dynex Technologies, Chantilly, VA) on a Molecular Devices SpectraMax Gemini spectrophotometer with excitation at 380 nm and emission at 460 nm (25°C for 30 min).

Comparison of quenched fluorescent substrates: Substrates with an amino-methyl-coumarin (AMC) leaving group were synthesized by commercial vendors. Hydrolysis of quenched substrates was measured using a synergy HI plate reader (BioTek Instruments, Winooski, USA) with excitation at 320 nm and emission at 460 nm (37°C for 60 min). The initial velocity was calculated from the initial linear part of the progress curve. The standard assay system consisted of TBS (25 mM Tris-HCl, pH 7.5, and 150 mM NaCl) with $CaCl_2$ (2 mM) and Tween-20 (0.1% w/v). Enzyme concentrations are indicated in the figure legends and the concentration of peptide substrates was from 0-1250 μM. A standard curve was prepared using AMC and molar concentrations of hydrolyzed products were calculated and the data fitted to Michaelis Menten kinetics using Graphpad Prism software (San Diego, CA) to determine K_m, k_{cat} and k_{cat}/K_m. The following enzymes were used; Human neutrophil elastase (Merck-Millipore, Olso, Norway), human thrombin (Sigma Aldrich, Oslo, Norway), **Low molecular weight urokinase (uPA) (Sekisui, Pfungstadt, Germany)**, **tissue plasminogen activator (tPA), (Actilyse, Boehringer Ingelheim, Germany)**, **Factor XIIa (Haemochrome Diagnostica, Essen, Germany),** bovine Factor Xa (NEB, Ipswich, MA, USA), Plasmin (in-house purified human plasma plasminogen activated with pro-uPA in a ratio of 100:1).

Structural analysis of FSAP-substrate complexes: A homology model of the FSAP serine protease domain was generated using SWISS-MODEL server with default settings (28). The C-terminal 261 residues of the FSAP protease domain sequence was modelled on tPA (PDB

ID: 1BDA) with which it has **a high** sequence identity (43% and a QMEAN value of 2.54). The 3D models of peptide substrates were built using BuildStructure tool in Chimera (29), and each amino acid was assigned with dihedral torsion angles predicted by ANGLORE (30). The peptide models were then docked onto the substrate-binding pocket of the FSAP model using Coot (31) and molecular clashes were refined. The preliminary FSAP-peptide models were submitted to the high resolution Rosetta FlexPepDock server with default settings (32). The Rosetta scores for the top 10 models were between -59.8 and -72.3 and the RMSD (root mean-square deviation) ranges between 3 and 10, indicating the close proximity of the models. The structures presented here are with the lowest RMSD value of 3-4, as they show a better fit with the protein in terms of steric clashes and RMSD value. The FSAP-peptide model structures were visualized and figures were prepared by using PyMOL Molecular Graphics System; Version 1.7.2.0 (Schrödinger, LLC).

Activation of FSAP in human plasma: Human citrated-plasma was used for these studies and activation was performed with either dextran sulphate (DS) or histones for 60 min at 37°C. Activation was also performed in the presence of inhibitors or antibodies as indicated in the figure legends. Thereafter, the enzymatic activity was determined by direct addition of fluorescent substrate to the plasma. Turnover of the fluorescent substrates was determined as described above.

RESULTS

Substrate specificity of FSAP using phage display: To determine the substrate specificity of FSAP against small peptide substrates a method based on phage display was used as described before (24). The phage library consisting of 7 amino acid peptides inserted in the biotinylated pIII protein was captured on beads and then released by proteolytic cleavage with FSAP. **After 3 rounds of panning of the library, 500 phage clones were screened in two**

independent experiments for cleavage by FSAP in the phage ELISA. From these, 45 individual phage clones that showed a 20%, or greater, phage release in the presence of FSAP were identified (Fig. 1A). The observed variation in cleavage in these experiments was not only due to different peptide sequences but also due to the variable phage titres as well as the degree of biotinylation in the different phage clones. To test the specificity of the cleavage by FSAP, 5 of the 45, phage clones were selected randomly and further characterized. These 5 clones demonstrated a concentration-dependent cleavage by FSAP (Fig. 1B). The cleavage of these same 5 phage clones could be blocked by aprotinin which is known to inhibit FSAP activity (Fig. 1C). 45 clones exhibiting bona fide cleavage by FSAP were sequenced and their alignment **(Fig. 2A)** indicated a preference for basic amino acids at the P1 and P2 positions, apolar amino acids at P3 and P4 positions and Ser at P1' position **(Fig. 2B).** The consensus sequence obtained was Val-Leu-Lys-Arg-Ser and the scissile bond is likely to be between the residues Arg-Ser or Lys-Arg.

Substrate specificity of FSAP using PS-SCL: We then used another random peptide library based method to consolidate these findings and to aid the development of selective synthetic substrates. This was done using a combinatorial library of synthetic peptide substrates each consisting of 4 amino acids and the fluorescent leaving group at P1' position. There was a strict specificity for basic amino acids (Arg > Lys) at the P1 position, whereas at the P2 position there was a preference for NLe>**Asn**=Ser=Ala=Met=**Lys**. In the P3 position there was a preference for basic residues followed by apolar residues (Lys>Arg=Tyr=Leu=Met) and in the P4 position there was no clear preference (Fig. 3). Although there was considerable degeneracy in terms of the consensus sequences deduced it was clear that a cluster of basic residues would be ideal cleavage sites for FSAP and thus overlaps to some extent with the prediction through the phage display experiment.

Pattern of cleavage by FSAP in large protein substrates: A few studies have reported FSAP cleavage sites in large protein substrates. Cleavage data for PDFG-BB (33), kininogen (34) TFPI (35), C5 and C3 (36), fibrinogen (37) and FSAP (38) was obtained experimentally. For uPA and FVII the predicted cleavage site for zymogen **activation** are shown. However, beyond the requirement for the basic amino acids Arg or Lys at the P1 site as well as a cluster of basic amino acids on the prime side no further pattern could be recognized (Fig. 4).

Identification of specific quenched fluorescent FSAP substrates: Based on the above analysis, and also by taking into account the sequence preferences of other proteases from the hemostasis system, we designed and tested fluorescent peptide substrates for FSAP. The substrate with the core sequence Ala-Lys-Nle-Arg showed the highest affinity for FSAP, whereas other substrates Ala-Leu-Lys-Arg > Val-Pro-Arg > Arg-Val-Arg-Arg > Gly-Gly-Arg showed decreasing affinity. Catalytic turnover also showed a similar pattern for the above tested substrates (Fig. 5A). Other plasma proteases were also tested to determine the specificity of the cleavage of this substrate by FSAP. FSAP was about 10-fold more efficient ($kcat/K_m$) than thrombin, plasmin, FXa, **uPA, tPA, FXIIa** and neutrophil elastase (for the latter data is not shown). **Factor XIIa, plasmin and thrombin could also cleave this substrate to some extent** (Fig. 5B). The kinetic data comparing proteases and substrates **from replicate experiments** is summarized in Table 1. The substrate Ala-Leu-Lys-Arg-AMC showed lower catalytic efficiency for FSAP but an even higher selectivity for FSAP over **FXIIa, thrombin as well as** other proteases **except plasmin (Fig. 5C and Table 1)**. These results confirm the specificity of the designed peptide substrate for FSAP.

Modelling studies to examine the docking of peptide substrates into the active site of FSAP: In the absence of any experimentally derived structure of FSAP, we modelled the serine protease domain of FSAP on that of tPA. The overall model of the FSAP serine protease

domain (SPD), based on tPA, consists of two six-stranded barrel-like sub-domains (Fig. 6A), as observed in other chymotrypsin family proteins (39). The two six-stranded barrel-like domains pack against each other at an interface, which forms the catalytic site. The catalytic triad residues Ser509 (c195), His362 (c57) and Asp411 (c102) show very good alignment with the catalytic triad residues of tPA (data not shown). **The numbers in parenthesis refer to the chymotrypsin numbering system.** The side-chains of the catalytic nucleophile Ser509 (c195) and His362 (c57) are in a configuration that allows the formation a hydrogen bond between them, with a distance of 2.9 Å between their respective O^{γ} and N^{ε} atoms (Fig. 6A).

In the peptide substrate (Val-Leu-Lys-Arg-Ser), identified by phage display, the scissile bond is likely to be between the residues Arg-Ser or Lys-Arg. In this model the Arg has a higher preference at P1 compare to Lys. P2 residue is preferentially Lys rather than Arg, whereas P3 and P4 residues are more diverse. On the other hand, the P1' residue is likely to be Ser rather than an aromatic amino acids. Thus cleavage is likely to be at the Arg-Ser bond as indicated in Fig. 2.

The peptide was docked into the active site of FSAP using Rosetta FlexPepDock server to further analyze the protein-peptide interaction (Fig. 6B). The peptide is stabilized by the coordination with the neighboring residues in the active site of the protease; the two side chain nitrogens (N1 and N2) of the Arg (P1) residue are stabilized in the S1 pocket by hydrogen bonds. The N1 coordinates with the side chain of Asp503 and main chain oxygen of the Glu532 residue with a distance of 3.1 and 2.8 Å, respectively. The N2 forms hydrogen bonds with the side chain oxygen of Thr504 (2.5 Å) and the main chain oxygen of Trp529 (2.5 Å) (Fig. 6C). On the contrary, the main chain oxygen of Arg shows relatively weak interaction (3.2 Å) with amide nitrogen of the Gly507 residue. The side chain of Ser (P1') interacts with the side chain nitrogen of Gln506 at a distance of 2.9 Å. All the interactions collectively hold the peptide substrate in the active site pocket of protease domain (Fig. 6C).

The P2, P3 and P4 residues fit in the pocket but do not show significant interaction with the neighboring amino acids.

Analysis of FSAP proteolytic activity in plasma using Ala-Lys-Nle-Arg-AMC: Nucleosome and histones are the only known physiological activators of **pro**-FSAP in plasma and in vivo (2). These are released from injured or inflamed tissue and alter FSAP activity in the plasma. We tested the turnover of our most sensitive FSAP substrate, Ala-Lys-Nle-Arg-AMC, in plasma activated with histones. **Kinetic studies showed that substrate turnover was activated almost immediately after addition of histones to plasma. This activation could not be inhibited by lepirudin, which inhibits thrombin, or by corn trypsin inhibitor (CTI) which inhibits FXIIa (Supplementary Fig. S1A). Kinetic studies on plasma from a subject homozygous for the MI genotype showed only basal substrate turnover and no increase in the presence of histones (Supplementary Fig. S1B). These results indicate that the substrate undergoes FSAP-specific turnover in plasma. The pattern of pro-FSAP activation by histones in the presence of inhibitors was the same whether we determined substrate turnover or if we measured FSAP α2 anti-plasmin complexes (40) as an indirect index of FSAP activation (Supplementary Fig. 2).**

Further studies were performed with dextran sulphate (DS), which activates FXIIa and the contact pathway, as well as pro-FSAP (9). Substrate turnover was about 20-fold higher in DS-treated plasma compared to histone-treated plasma which confirms that **FXIIa** also cleaves this substrate as was observed with purified enzymes (Fig. 7A). **CTI completely inhibited the activation of proteolytic activity by DS, but it did not influence substrate cleavage in histone-activated plasma.** Aprotinin which inhibits FSAP as well as the proteases in the contact and fibrinolysis pathway inhibited substrate activation with both activators whereas lepirudin, which inhibits thrombin, had no influence on **the effect of** either activator. A specific FSAP-inhibiting Mab-570 inhibited the turnover of the fluorescent

substrate in histone-activated plasma but not in DS-activated plasma whereas a control Mab had no affect at all (Fig. 7A). This indicates that histones specifically activate the proteolytic activity of FSAP in plasma, as determined by Ala-Lys-Nle-Arg-AMC turnover, independently of the contact pathway and thrombin activation.

The above described substrate turnover assay was also compared with two other assays currently used to measure FSAP activity; the pro-uPA activation assay and the FSAP-inhibitor complex assay which is an indirect indicator of FSAP activity. For these tests we mixed FSAP-depleted plasma with defined amounts of purified FSAP. The substrate turnover assay could detect the activity of 75 nM exogenously activated plasma but it should be noted that there is probably a rapid inhibition of exogenously added FSAP by endogenous plasma inhibitors so this value does not reflect the true sensitivity of this assay. The assay which measured pro-uPA activation after immunocapture of FSAP could detect about 25-fold lower levels of active FSAP and this was also the case with the FSAP-α2 anti-plasmin complex ELISA (Supplementary Fig. 4). In the pro-uPA activation assay the immobilization of FSAP and the extensive washing seems to reduce the influence of endogenous inhibitors leading to a higher sensitivity of FSAP activity detection.

In order to further consolidate the use of this substrate in determining the activity of FSAP in plasma we compared normal plasma with FSAP-deficient plasma as well as plasma from a subject that is homozygous for the MI-SNP. All three plasmas could be activated equally well by DS but histones failed to induce substrate turnover in FSAP-deficient plasma as well as in MI-plasma (Fig. 7B). Thus, in conjunction with a specific FSAP substrate and an inhibiting antibody it will be possible to accurately measure the activity of FSAP in a complex biological setting such as plasma.

DISCUSSION

Although human genetic studies and data from knockout mouse models show that FSAP may play important roles in vivo, to date, no information is available as to the nature of the substrate specificity of FSAP. In order to provide answers to some of these questions we have carried out a systematic study of FSAP substrates and **have** design**ed** substrate**s** that can be used to measure the proteolytic activity of FSAP in the plasma.

The phage display and PS-SCL method allows a hypothesis-independent prediction of the peptide substrate around the cleavage site of a protease. However it should be noted that small peptide substrates behave differently to protein substrates and a direct extrapolation from peptide to protein is not always possible. At least, for the proteases from the hemostasis system, substrate interactions with exosites or other regulatory domains in the enzyme play a major role and this factor is missing in the approaches used here. The simultaneous use of both methods allows us to overcome limitations of a single method e.g., the PS-SCL approach is not useful for analysing the cooperative interactions between the residues at the different positions or to reach any conclusions about the amino acids on the prime side of the cleaved bond, something which is possible with phage display. **One of the goals of this study was to identify a sequence motif that could be used to describe FSAP substrates. However, the phage display, PS-SCL approach and alignment of protein substrates did not uncover a specific pattern except** that FSAP prefers a cluster of basic amino acids on the non-prime side of the cleavage site. **Within such a basic cluster there seems to be a preference for a dibasic motif at the cleavage site.**

Our modelling studies show that it is possible **to predict the configuration of the** active site of FSAP based on tPA in a realistic manner. The P1 Arg shows the strongest interaction compared to Lys in the S1 pocket. This interaction in FSAP is quite comparable to the interaction of the Arg residue of the dansyl-Glu-Gly-Arg-chloromethylketone inhibitor with tPA (39). The overall fit of the Val-Leu-Lys-Arg-Ser peptide shows a similar backbone

orientation as in tPA-inhibitor binding mode. One the other hand, replacement of P1 residue with Lys shows relatively weaker interaction which matches the results from the phage display studies. Thus, the modelling confirms that the identified peptide substrate showed a favorable electrostatic interaction and geometric compatibility with the active site of FSAP.

In designing the FSAP activity probe we also considered the known substrate specificity of major proteases from the hemostasis system. Using PS-SCL it was reported that thrombin prefers Ala-Tyr-Pro-Lys-ACC, plasmin prefers Ala-Met/Gln-Phe/Tyr-Arg-ACC, FXa prefers Ala-Arg-Phe/Gly/Ser-Arg-ACC and urokinase prefers Ala-Gly/Thr/Ser-Ala/Gly/Ser-Arg-ACC (41). Based on our own results, it was evident that P1 should be Arg, P2 should be basic or Nle based on phage display and PS-SCL respectively and P3 should be a basic or hydrophobic amino acid based on both methods. To diminish cleavage by other proteases we selected Ala-Leu-Nle-Arg-AMC and Ala-Leu-Lys-Arg-AMC for further analysis. Of the 5 substrates tested Ala-Leu-NLe-Arg-AMC showed the best kinetic properties against FSAP **and high selectivity over other enzymes except FXIIa, plasmin and thrombin**. **FSAP had lower activity against Ala-Leu-Lys-Arg-AMC but this substrate had the best selectivity against other proteases except plasmin. Since many more proteases exist in plasma, studies with isolated enzymes need to be expanded in the future**. **Further modifications could be made to the existing peptide, e.g., by using amino acid derivatives, to further enhances the selectivity and specificity for FSAP.**

Assays in whole plasma that was activated with the known FSAP activator, histone, confirmed the utility of this probe in a complex setting. A specific monoclonal antibody that inhibited FSAP could block substrate turnover and FSAP-deficient plasma and homozygous MI-FSAP plasma **exhibited no activity. Thus, the substrate was clearly cleaved in a FSAP-dependent manner. Use of CTI and lepirudin showed that FXIIa and thrombin enzymes were not involved in the turnover of the substrate. These results are also**

important for deciphering the role of histones in the plasma since FSAP activation may influence the extrinsic pathway (22).

Unexpectedly, the substrate was also activated in dextran sulphate activated plasma that would indicate cleavage by enzymes from the contact pathway **such as FXIIa**. **CTI, which blocks FXIIa, completely reduced substrate turnover completely which was unexpected considering that the activation of FSAP by dextran sulphate should remain intact in the presence of CTI (9). This absence of activity is most likely to be due to the fact that polyanions have been shown to be cofactors for complex formation between FSAP and various SERPINS found in plasma (42). Thus, dextran sulphate activates FSAP which is then rapidly inactivated by a dextran sulphate-driven inhibitory mechanism resulting in no net change in activity.**

Through the use of an FSAP inhibitory antibody as well as CTI and hirudin it will be possible to measure FSAP activity in the plasma using this probe with a high degree of reliability. This possibility to directly measure FSAP activity in the plasma vastly supersedes, **in terms of simplicity**, the current method requiring FSAP immunocapture followed by measuring the conversion of pro-uPA to active uPA using chromogenic substrates (36). **Although this method, as well as the FSAP-inhibitor complex ELISA, can also measure FSAP activity with high sensitivity, only the fluorescent substrate allows a direct real-time kinetic measurement of changes in FSAP activity.**

Future development of FSAP as a therapeutic target will require the development of specific inhibitors. Based on the substrate specificity data it is possible to attach an inhibitory group, such as chloromethylketone, instead of the AMC leaving group in order to design a specific inhibitor. Although we did not design any specific inhibitors of FSAP in this study we did test a commercially available inhibitor for furin (Kex2) (43), decanoyl-Arg-Val-Lys-Arg-CMK that also is rich in basic amino acids and quite similar to the FSAP substrates identified in this study. This inhibitor could inhibit the proteolytic activity of FSAP with

comparable kinetics (IC_{50}= 0.155 ± 0.032 μM, n=6) to aprotinin (IC_{50}= 0.066 ± 0.013 μM, n=6) (data not shown). This detailed study on the characterization of FSAP substrates will provide the basis for developing inhibitors, identifying novel substrates and for designing activity-based probes for diagnostic purposes.

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Legends to Figures

Figure 1. Identification of phage clones cleaved by FSAP: After 3 rounds of panning 500 individual phage clones were selected for further analysis. These were immobilized on neutravidin-coated plates and incubated in the absence or presence of FSAP (30 nM) or control buffer for 18 h at 37°C. Thereafter the wells were washed and developed with peroxidase-coupled anti phage Mab M13. Loss of absorbance indicates cleavage and release of phage by FSAP. (A) Results are shown for 45 clones that showed a greater than 20% cleavage by FSAP compared to buffer control. These results represent 2 independent replicates of the experiment performed in singlet. (B) Five positive clones from (A) were further tested using different concentrations of FSAP (**0-150 nM**) for 4 h at 37°C. (C) The same five clones from (B) were incubated with FSAP (**50 nM**) in the absence or presence of aprotinin (50 μg/ml). Experiments were performed in singlet and similar results were obtained in 2-independent replicates of the experiment **(mean ± SEM, n=2)**.

Figure 2. Sequence of preferred FSAP peptide substrate as determined by phage display: (A) 45 phage clones cleaved by FSAP were sequenced and the variable insert peptide sequence was aligned by MafftWS. **(B)** The fold enrichment of amino acids in each position is represented as an iceLogo. Dashed lines indicate the probable scissile bond based on the modelling studies below.

Figure 3. Sequence of preferred FSAP substrate as determined by PS-SCL: An arrayed synthetic tetrapeptide library with quenched fluorescence was subjected to proteolysis by FSAP (**30 nM**). **(A)** Y-axis represents fluorophore released per time unit converted to a % of maximum for the best amino acid at the specified position in the tetrapeptide. X-axis indicates the amino acid held constant at each position respectively (P1-P4) in the tetrapeptide (n

represents norleucine). Each bar represents the mean ± SEM (n=3). **(B) The fold enrichment of amino acids in each position is represented as an iceLogo.**

Figure 4: Comparison of the sequence of native macromolecular FSAP substrates: Sequences of known FSAP protein substrates were aligned at the cleavage site (A) and are depicted as an iceLogo (B). Dashed line indicates the scissile bond between P1 and P1'. Cleavage data for all proteins was obtained experimentally except for uPA and FVII **where** the predicted cleavage site for **zymogen** activation is shown.

Figure 5: Comparison of the kinetic properties of fluorescent peptide substrates: (A) The kinetic properties of FSAP using various peptide substrates were determined using Michaelis-Menten analysis. The concentration of FSAP was 20 nM and the substrate concentration was varied between 0-1250 μM and the initial velocity was measured. **Experiments were performed in duplicate and data is shown as mean. Enzyme kinetic** analysis was performed using Graphpad Prism. (B) Same as above except that different plasma proteases were tested using the lead substrate, Ala-Lys-Nle-Arg-AMC. Enzyme concentrations were as follows: FSAP (18 nM), thrombin (17 nM), plasmin (10 nM), FXa (15 nM), **uPA (20 nM), tPA (20 nM), FXIIa (7 nM).** (C) **Same as above except that different plasma proteases were tested against the substrate Ala-Leu-Lys-Arg-AMC. The complete kinetic data, including the number of replicates is in Table 1.**

Figure 6: The FSAP-peptide complex: (A) The model of the serine protease domain of FSAP is shown in blue and the catalytic triad residues are show as sticks where the nitrogen atoms are blue and the oxygen atoms red. The residues Ser509 (c195), His362 (c57) and Asp411 (c120) are shown in a zoomed configuration. The numbers in the brackets indicate the chymotrypsin numbering system. The O^{γ} of Ser509 and N^{ε} atom of His362 form a

hydrogen bond at a distance of 2.9 Å. (B) The overall model FSAP- Val-Leu-Lys-Arg-Ser complex where FSAP is shown as a solid surface and the peptide is shown as a ball-stick model. In FSAP, the positively charged residues and the negatively charged residues are colored as blue and red, respectively. For the peptide, the carbon atoms are shown in orange, the nitrogen atoms in blue, the oxygen atoms in red. (C) The active site residues of FSAP are shown in blue sticks and the Val-Leu-Lys-Arg-Ser peptide is shown in orange sticks and the interacting residues are numbered accordingly. The yellow dotted lines indicate the hydrogen bonds and the numbers indicate the corresponding distances in Å.

Figure 7: Measurement of FSAP activation in the plasma: (A) Normal human plasma (1:20 dilution) was incubated with corn trypsin inhibitor (CTI, 50 μg/ml), aprotinin (50 μg/ml), lepirudin (25 μg/ml), Mab570 or control Mab (100 μg/ml), in addition to histones (100 μg/ml) or dextran sulphate (DS) (100 μg/ml). Ala-Lys-Nle-Arg-AMC substrate (40 μM) was added and the proteolytic activity monitored for 1h at 37°C. (B) Same conditions as above except that normal plasma was compared to plasma from a subject homozygous for the MI-SNP as well as FSAP-deficient human plasma which was prepared by adsorption over an FSAP-**antibody** column. Results are mean ± SD of duplicate wells and similar results were obtained in 2 independent experiments.

Table 1: Comparison of the kinetic properties of different substrates and proteases. All values are mean ± SEM. **Numbers in parenthesis indicate the number of independent experiments. Top panel depicts comparison of different substrates against FSAP. Middle panel and lower panel depict comparison of different enzymes against Ala-Lys-Nle-Arg-AMC and Ala-Leu-Lys-Arg-AMC respectively.**

FSAP	K_m **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m
Ac-Ala-Lys-Nle-Arg-AMC (n=3)	45 ± 12	71 ± 24	1.58
Ac-Ala-Leu-Lys-Arg-AMC (n=3)	96 ± 30	40 ± 9	0.42
Boc-Val-Pro-Arg-AMC (n=3)	578 ± 183	38 ± 9	0.12 ± 0.06
Boc-Arg-Val-Arg-Arg-AMC (n=3)	very high	unreliable	unreliable
Z-Gly-Gly-Arg-AMC (n=3)	very high	unreliable	unreliable

Ac-Ala-Lys-Nle-Arg-AMC	K_m **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m
FSAP (n=3)	45 ± 12	71 ± 24	1.58
Thrombin (n=3)	298 ± 6	49 ± 15	0.16
Plasmin (n=3)	537 ± 142	58 ± 17	0.11
FXa (n=3)	137 ± 35	3.5 ± 4	0.025
uPA (n=1)	1434	5.6	0.004
tPA (n=1)	621	2.0	0.003
FXIIa (n=4)	355 ± 96	103 ± 28	0.29

Ac-Ala-Leu-Lys-Arg-AMC	K_m **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m
FSAP (n=3)	96 ± 30	40 ± 9	0.42
Thrombin (n=2)	223 ± 27	0.6 ± 0.2	0.002
Plasmin (n=3)	1353 ± 317	71 ± 29	0.052
FXa (n=2)	118 ± 9	1.2 ± 1.2	0.01
uPA (n=5)	974 ± 139	9 ± 2.2	0.009
tPA (n=5)	346 ± 32	0.3 ± 0.14	0.0009
FXIIa (n=3)	193 ± 19	0.4 ± 0.04	0.002

What is known about the topic?

1. A role for FSAP in thrombosis, stroke, cancer, atherosclerosis and liver cancer is predicted.

2. No information is available about the substrate specificity of FSAP.

3. Sensitive and specific assays for FSAP activity in plasma are not available.

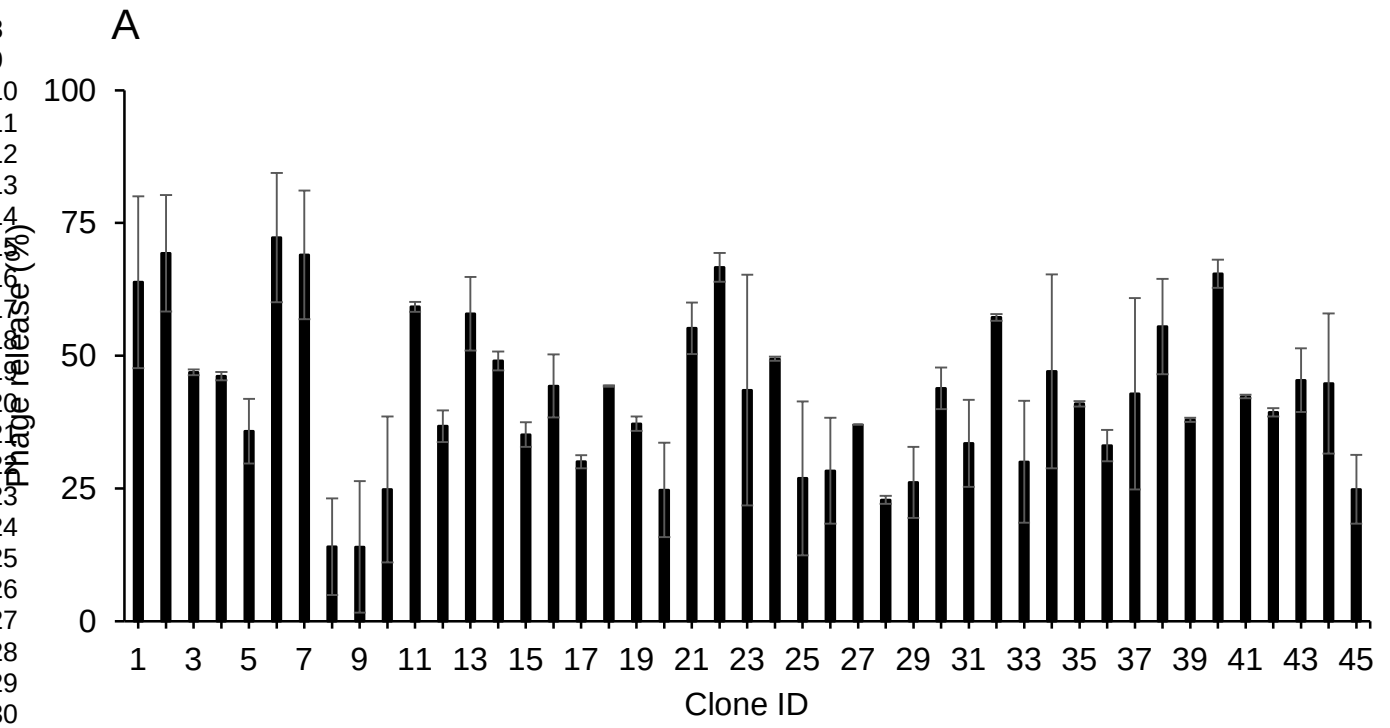
What does this paper add?

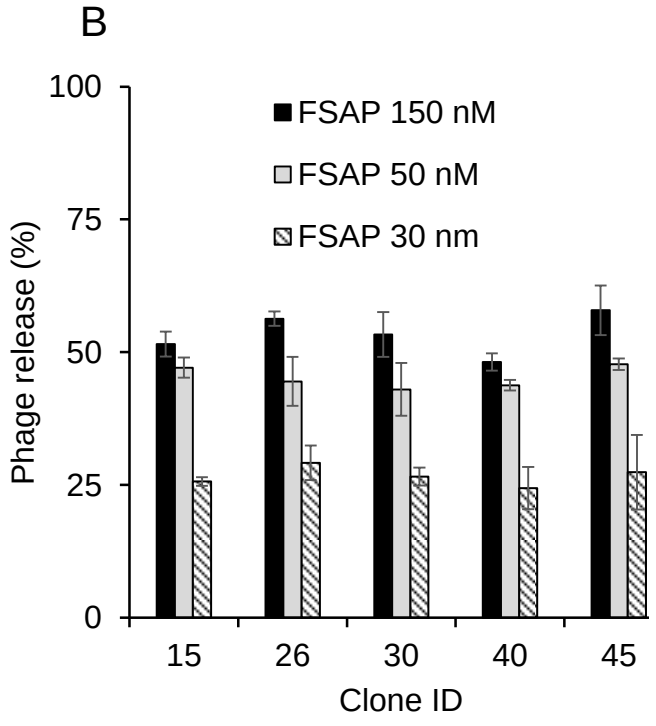
1. The substrate specificity of FSAP is characterized in detail.

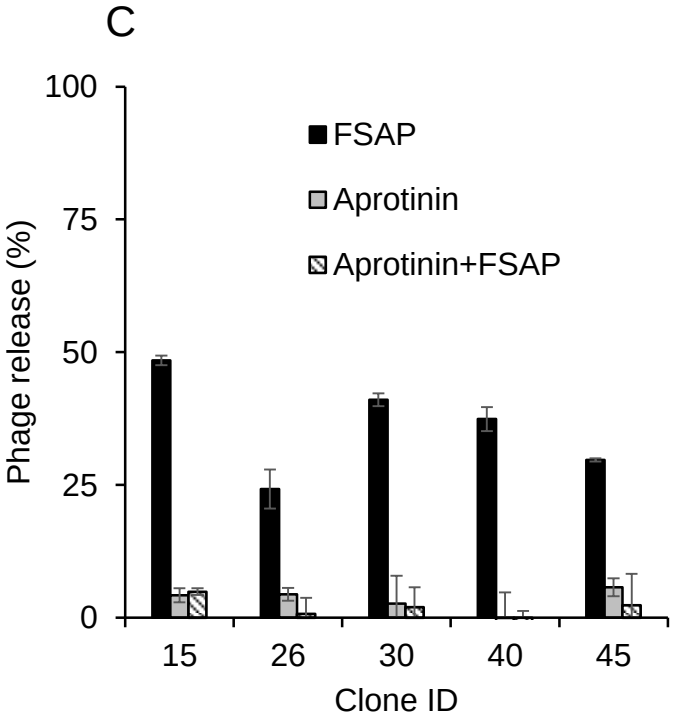
2. A novel substrate was designed to measure FSAP activity in plasma.

3. **Histone-mediated activation of pro-FSAP in plasma was demonstrated with this substrate.**

Kara et al. Fig.1





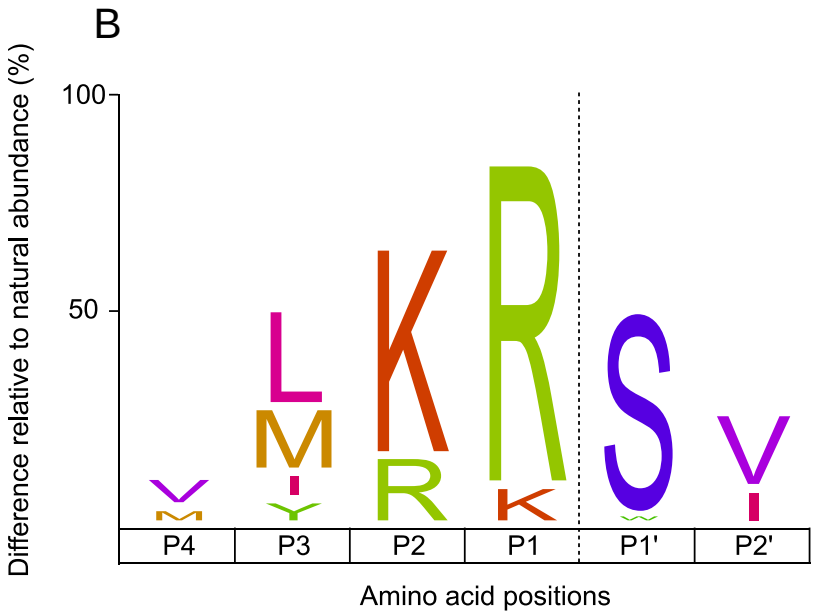


Kara et al. Fig.2

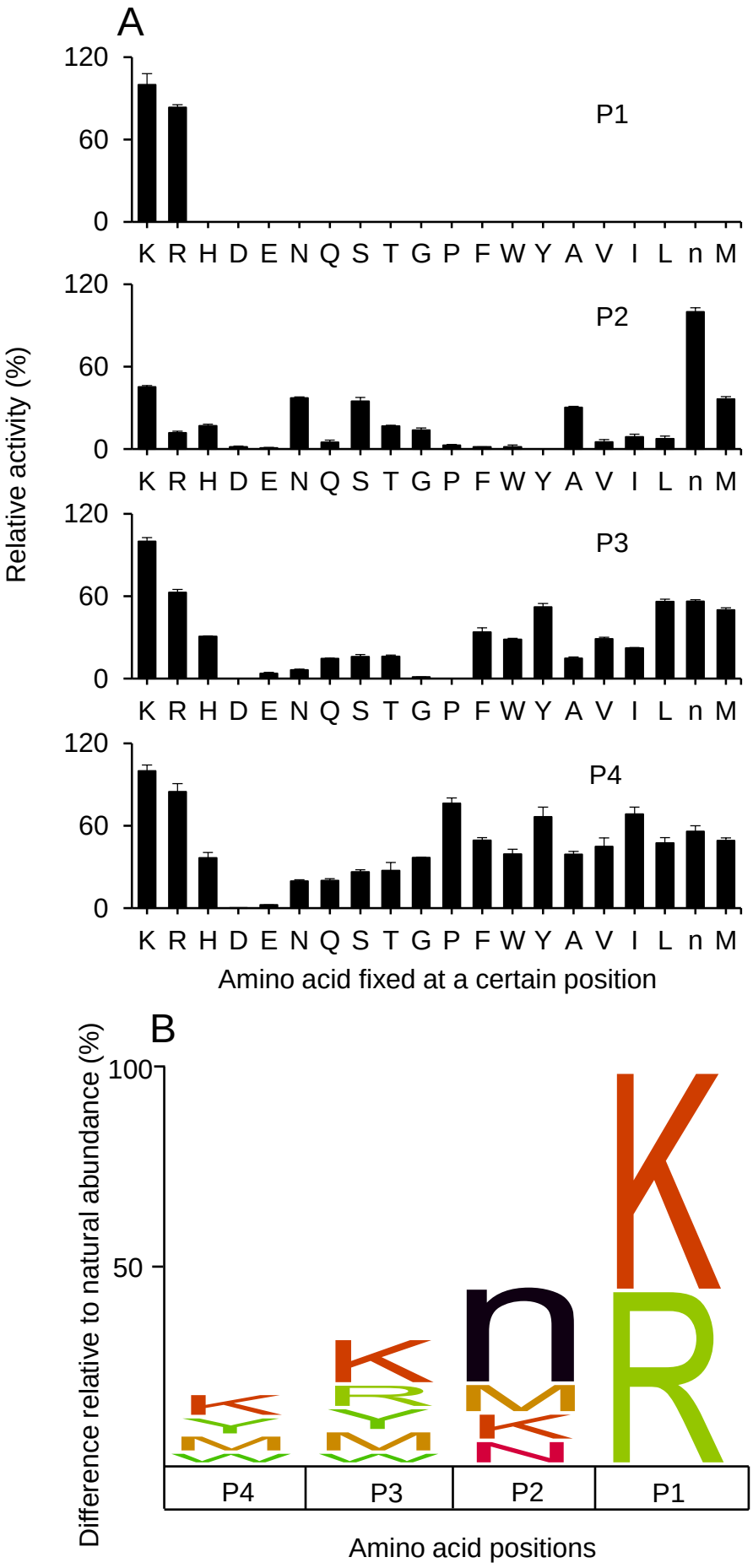
A

```
Clone1    --TPRKRSA--
Clone2    ----LKRTLLA
Clone3    --WVKKRWA--
Clone4    -TRVIKKS---
Clone5    ---VNTRSVK-
Clone6    --RPVKRSV--
Clone7    ----LKRSVLH
Clone8    ----PRRSYKQ
Clone9    --SLLKRTS--
Clone10   ----YKRSWAR
Clone11   -ARIIKKS---
Clone12   --LPLKRSY--
Clone13   ----YKRSISH
Clone14   --INYKRSF--
Clone15   ----HPRIVVK
Clone16   ----MMRSVML
Clone17   --QILMKKV--
Clone18   ----MKRWVRP
Clone19   ---VLKRSVL-
Clone20   --QMLKRYS--
Clone21   -AKPMKRL---
Clone22   ----LRRSIAR
Clone23   ----IRRSLLK
Clone24   ---VLVKSVL-
Clone25   ----LARSVRA
Clone26   ----IRRSLLK
Clone27   --NWVKKRR--
Clone28   --HVLRKSA--
Clone29   --SMLKRFV--
Clone30   --PIIRRSV--
Clone31   --IPKARAM--
Clone32   VPYKMRR----
Clone33   ---RHARSLL-
Clone34   -YKTLKRN---
Clone35   --VHIKRKA--
Clone36   ----YMRSISV
Clone37   --PTMRKRH--
Clone38   THATHRR----
Clone39   ----MTRSIKP
Clone40   --RPLKRTA--
Clone41   TKPMMRR----
Clone42   ----LRRSIAW
Clone43   --LHRKRS---
Clone44   --LALKRT---
Clone45   ----KKRSLFK
```

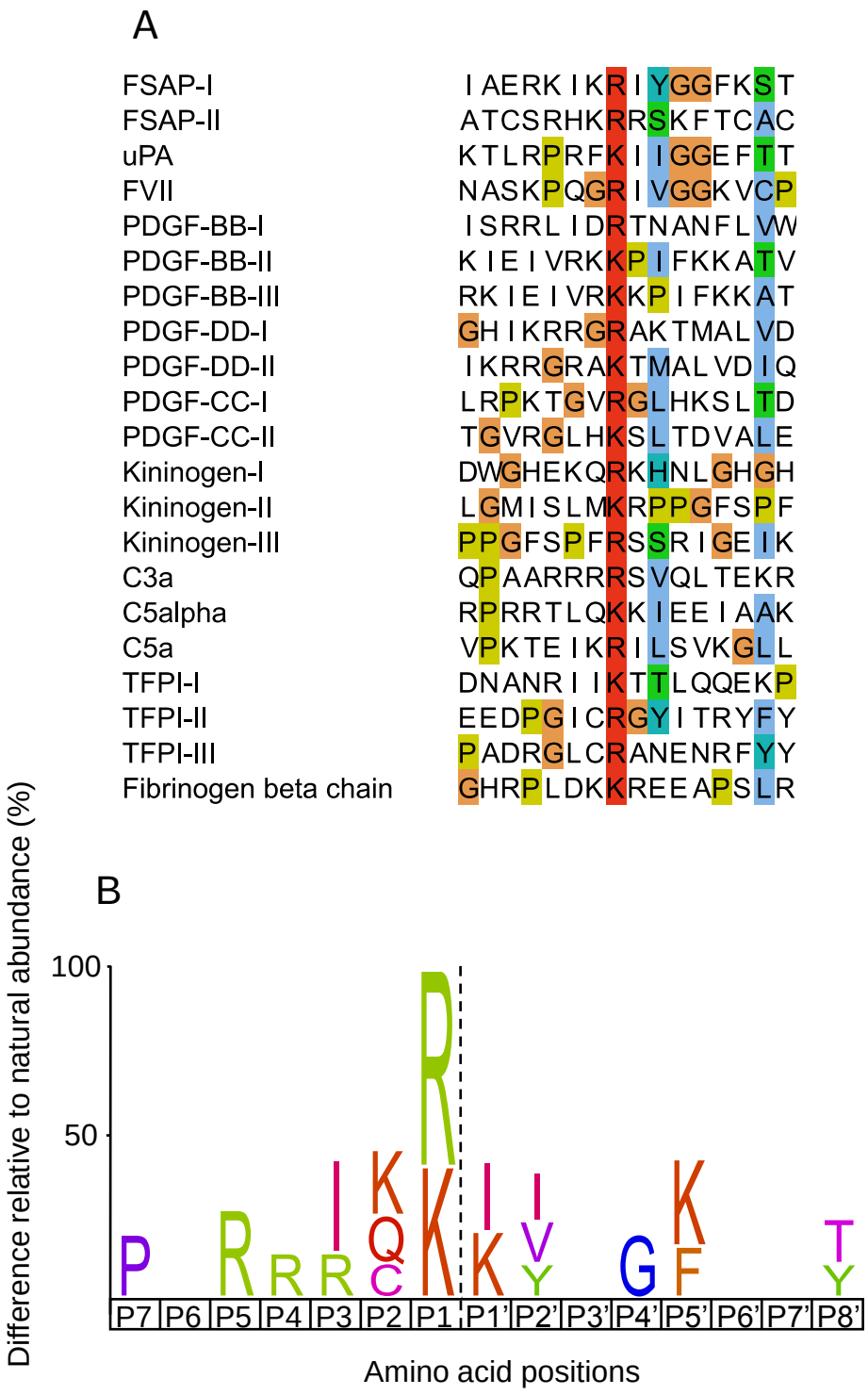
B



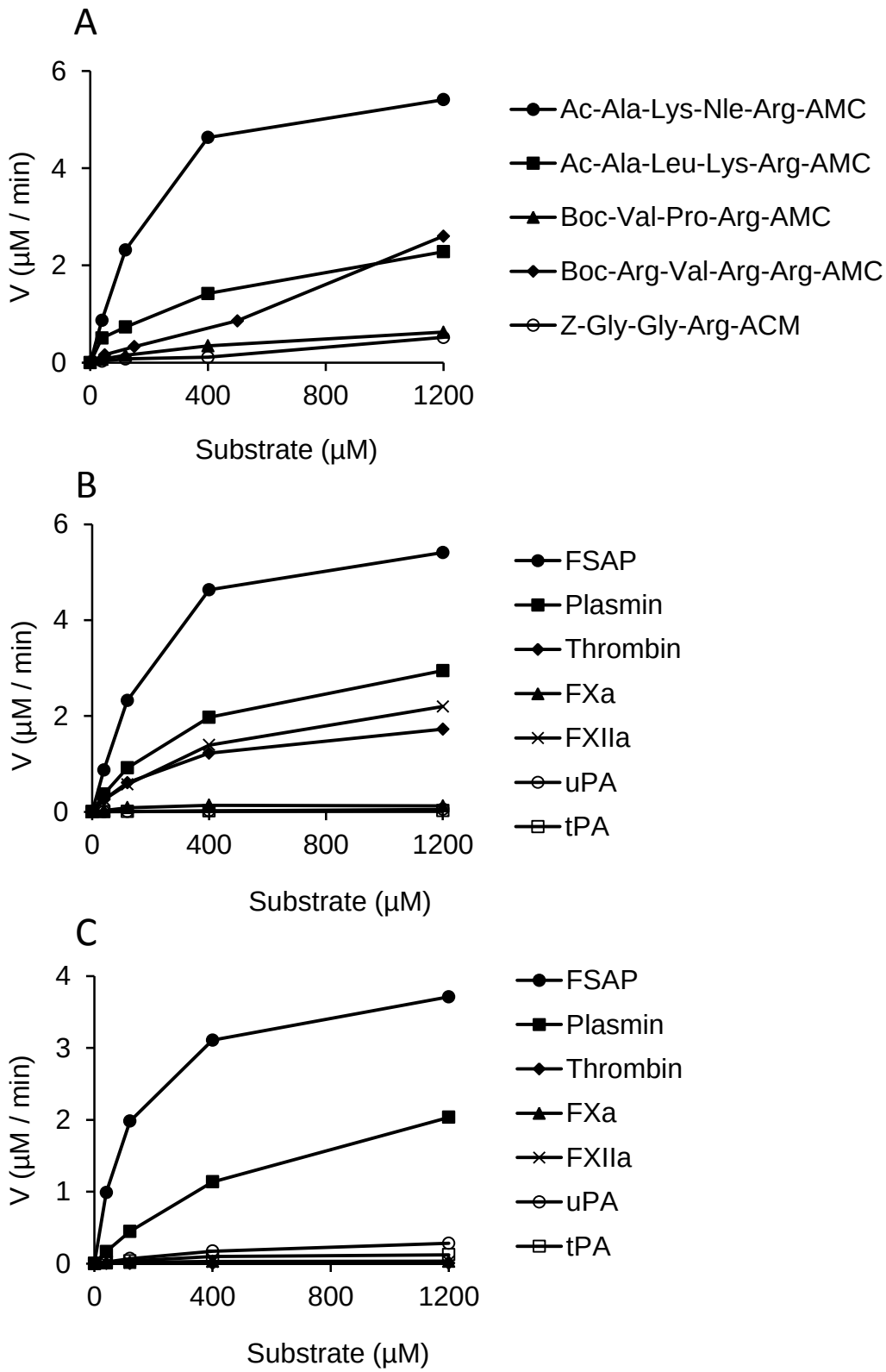
Kara et al. Fig.3



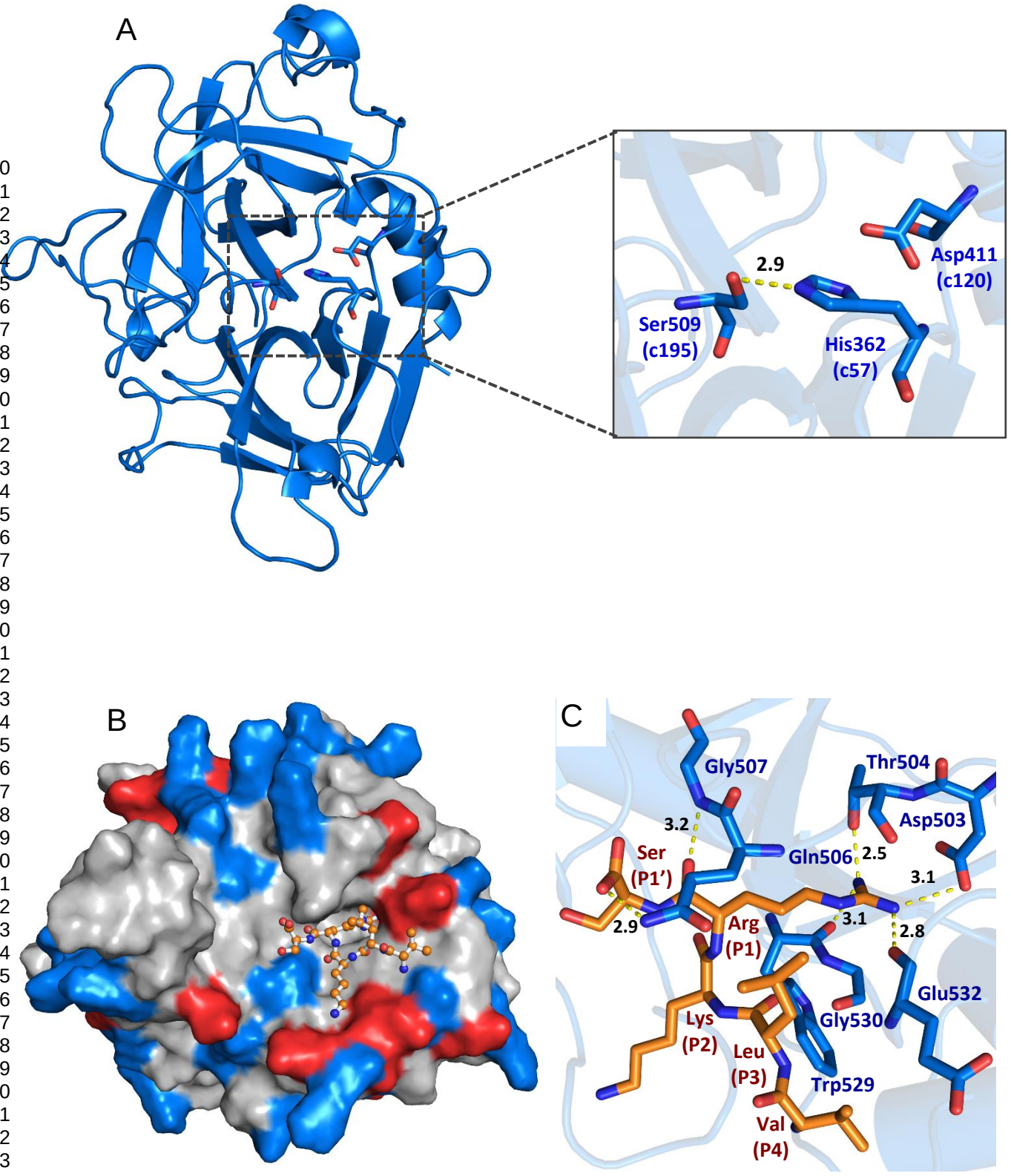
Kara et al. Fig.4



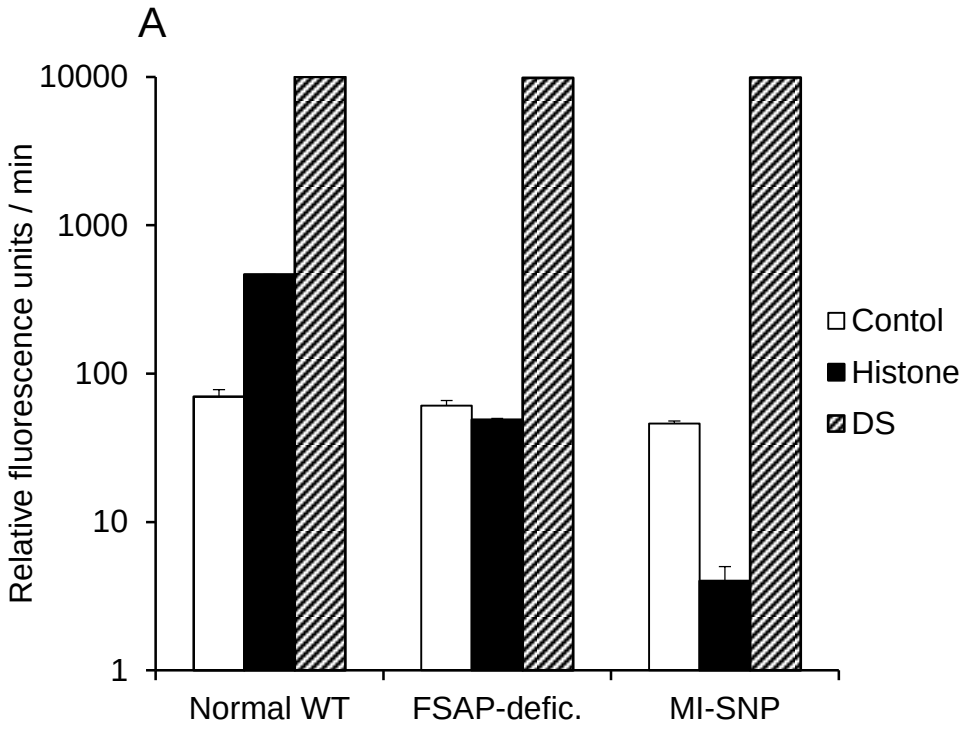
Kara et al. Fig.5

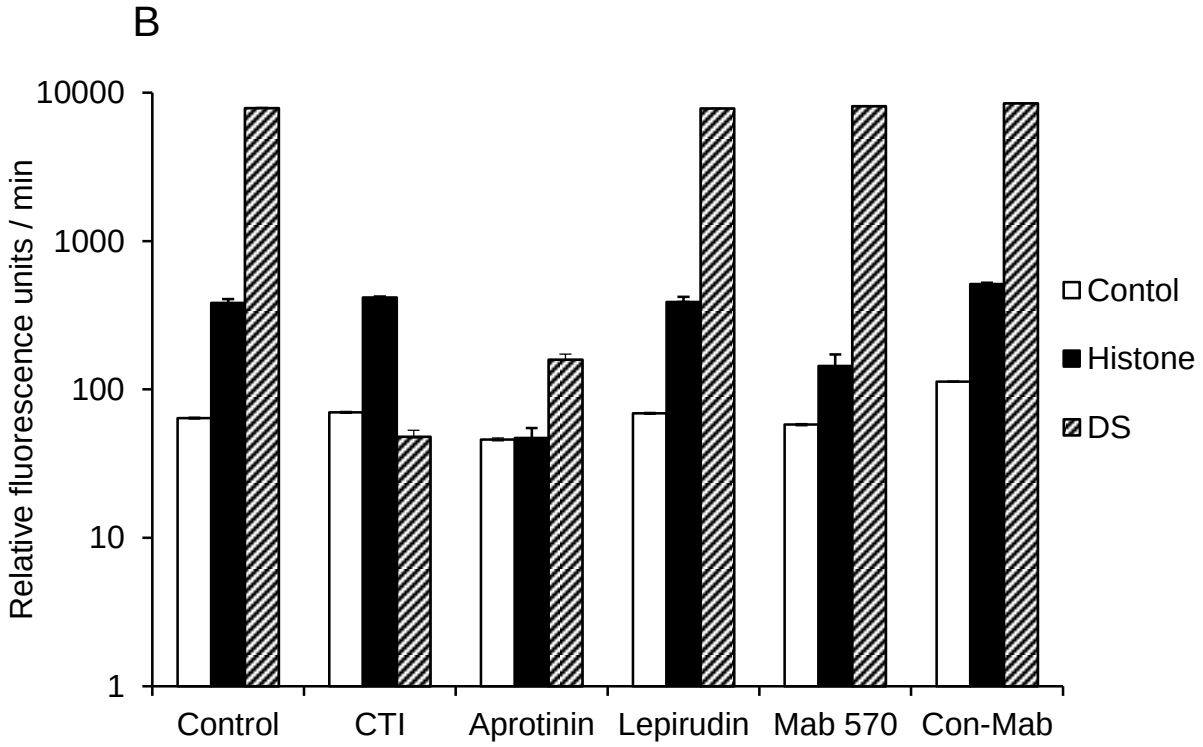


Kara et al, Fig. 6



Kara et al, Fig. 7





Thrombosis and Haemostasis

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