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Missense mutations in the gene encoding prothrombin corresponding to Arg596 cause antithrombin resistance and thrombomodulin resistance

（プロトロンビン Arg596 におけるミスセンス変異は

アンチトロンビン抵抗性とトロンボモジュリン抵抗性を引き起こす）

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主 論 文 の 要 旨

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【緒言】

静脈血栓塞栓症は生理的抗凝固因子の欠損症・異常症などの先天的要因と、加齢、肥満、長期臥床、妊娠、悪性腫瘍などの後天的要因が重なり発症する複雑な多因子性疾患である。

アンチトロンビン (antithrombin: AT) は、生体内でトロンビンなどのセリンプロテアーゼと 1 対 1 で結合することでその凝固活性を抑制する重要な抗凝固因子である。2012 年に当研究室は、AT 抵抗性により血栓症の原因となり得るプロトロンビン遺伝子 (*F2*) のミスセンス変異、プロトロンビン Yukuhashi 変異(c.1787G>T, p.Arg596Leu) を報告した。本変異型プロトロンビンの活性型である変異型トロンビンは野生型トロンビンと比較して凝固活性がやや低下している一方で、AT との複合体形成能が大きく損なわれていた。

また、トロンビンはトロンボモジュリン (thrombomodulin: TM) と結合することで凝固活性を失い、さらにはプロテイン C (PC) を活性化して活性化 PC (activated PC: APC) を産生する抗凝固的なはたらきに転じることから、TM も生理的なトロンビンの制御に重要である。我々はこれまで、596Leu 変異型トロンビンが TM による凝固活性制御にも抵抗性を示すことを明らかにしてきた。

ゲノム遺伝子上の一塩基置換はヒト生体内で一定頻度に発生する。本研究では *F2* での Arg596 コドン (CGG) の一塩基置換により生じうる 596Leu (CTG) 以外のミスセンス変異体(596Gln (CAG)、596Trp (TGG)、596Gly (GGG)、596Pro (CCG))が AT あるいは TM による抗凝固作用に及ぼす影響を評価した。

【方法】

596Gln、596Trp、596Gly、596Pro 変異型プロトロンビン発現ベクターを作製し、各変異型発現ベクターをヒト胎児腎由来 HEK293 細胞にリン酸カルシウム法にて遺伝子導入したのち、G418 による薬剤選択により安定発現細胞株を得た。各細胞株の培養上清・細胞溶解液のウェスタンブロッティング解析を行った。ビタミン K 含有無血清培地にて 24 時間培養して得られた培養上清を限外濾過にて濃縮後、ELISA により含まれるプロトロンビン量を定量し、各組換え型プロトロンビンをプロトロンビン欠乏血漿に添加した擬似患者血漿を検体として、凝固一段法ならびに合成基質二段法にて各変異型プロトロンビンの凝固機能を解析した。

各変異型プロトロンビン由来トロンビンの AT による不活化解析を実施した。十分に活性化した各トロンビン検体に AT を混和し、一定時間後に残存トロンビン活性を決定した。次に、各変異型トロンビンの TAT 形成能を評価した。十分に活性化した各トロンビン検体にヘパリン存在下／非存在下で AT を混和したのち、様々な時間で形成された TAT 量を ELISA により定量した。また、各変異型プロトロンビンのトロンビン活性化相とその不活化相を評価するため、トロンビン生成試験 (thrombin generation assay: TGA) を実施した。

各変異型プロトロンビン由来トロンビンの TM による不活化解析を行った。十分に活性化した各トロンビン検体に、組換え型可溶性 TM (sTM) を添加して 1 分後に精製ヒトフィブリノゲンを混和し、凝固時間法により残存トロンビン活性を測定した。次に、表面プラズモン共鳴 (surface plasmon resonance: SPR) 解析を用いて各変異型トロンビンの TM 結合量を評価した。

また、各変異型トロンビンの APC 産生能を評価した。十分に活性化した各トロンビン検体に sTM と精製ヒト PC を混和し、1 時間後に特異的発色性合成基質 S-2366 を添加して初速度法にて APC 活性を測定した。なお、活性測定直前にトロンビン阻害剤を添加し、残存トロンビン活性を完全に阻害した。さらに、血管内皮細胞 PC 受容体 (endothelial cell PC receptor: EPCR) 存在下での各変異型トロンビンの APC 産生能を評価した。すなわち、十分に活性化した各トロンビン検体と精製ヒト PC を、ヒト血管内皮細胞由来培養細胞（EAhy926 細胞）の培養液中に添加し、3 時間後に培養液を回収し APC 活性を測定した。

【結果】

Arg596 変異型組換え型プロトロンビン安定発現細胞株を樹立し、細胞溶解液ならびに培養上清のウェスタンブロッティング解析を行った結果、596Gln、596Trp、596Gly 発現株では野生型と同等のプロトロンビン産生が確認された。596Pro では、細胞溶解液・培養上清ともにほとんど検出されなかったプロトロンビンが、プロテアソーム阻害剤存在下で培養すると細胞溶解液には検出された。596Pro は培養上清にほとんど分泌されないため、以降の実験対象から除外した。Arg596 での各変異型プロトロンビンは様々な値の凝固活性を示したが、合成基質二段法での活性値は凝固一段法より一様に高値であった。

AT によるトロンビン不活化解析では、野生型トロンビンが継時的に不活化され AT 混和後 30 分で 15%程度まで不活化されたのに対して、各変異型トロンビンは 30 分後でも 80%以上のトロンビン活性残存率が保持された。また、野生型トロンビンはヘパリン非存在下で TAT 形成量が継時的に上昇したが、各変異型トロンビンは AT 混和後 60 分でもほとんど TAT が形成されなかった。TGA では、トロンビン活性持続時間を示す Start tail が、Arg596 での各変異型プロトロンビンにおいて野生型の 2 倍以上延長していた。

TM によるトロンビン不活化解析では、TM 濃度 25 μg/mL のとき野生型トロンビン活性が 16%まで低下したのに対して各変異型トロンビン活性残存率は 37~54%に保持された。一方、SPR 解析における各変異型トロンビンの TM 結合量は、野生型のそれと比較して低値であった。SPR 解析により算出された各変異型トロンビンと TM との解離定数は、トロンビン不活化解析結果を支持するものであった。EPCR 存在下・非存在下ともに、野生型／各変異型トロンビンの APC 産生能の差は各変異型プロトロンビンの凝固比活性の差に比例していた。

【考察】

本研究では、プロトロンビンの Arg596 コドンにおける一塩基置換により生ずるミスセンス変異体 (596Gln、596Trp、596Gly、596Pro 変異型プロトロンビン) の AT および TM による抗凝固作用に及

ぼす影響を評価した。596Pro 変異型プロトロンビンは、安定発現細胞株の細胞内・培養上清中にほとんど検出されなかったが、プロテアソーム阻害剤処理により細胞溶解液にはプロトロンビンが出現したことから、596Pro 変異が生体内で生じた場合、変異型プロトロンビンは細胞内でプロテアソーム系にて分解され、血中に分泌されないことが示唆された。また、凝固機能測定ではすべての野生型／変異型プロトロンビンで合成基質二段法では凝固一段法より高い測定値を示したが、これは凝固一段法の基質であるフィブリノゲン分子と比較して合成基質二段法で用いた発色性合成基質 S-2238 は非常に小さな分子であるため、野生型／変異型に関わらずトロンビンの活性中心に近づきやすいことが理由と考えられる。さらに、本研究の実験において 596Gln、596Trp、596Gly 変異型トロンビンは AT により不活化されにくく、同時に TM にも不活化されにくいことが示されたことから、これらの遺伝子変異が生体内で生じた場合、各変異型トロンビンは生理的制御機構に抵抗し、そのトロンビン活性を持続して血栓症を引き起こしやすくなることが推測される。

プロトロンビン Arg596 コドン(CGG)は一塩基置換のホットスポットである CpG 配列を含むが、CpG 配列ではシトシンがチミンに置換し得ることが知られており、本研究で解析した 596Gln、596Trp はシトシンからチミンへの置換により生ずる。本研究の遂行中にセルビア人家系で 596Gln 変異が、イタリア人家系で 596Trp 変異が発見され、いずれも家族性の静脈血栓塞栓症を発症したことが報告され、すなわち、Arg596 ミスセンス変異は人種を問わず生じ、血栓症の原因となりうることが強く示唆された。

【結語】

本研究では、プロトロンビン Arg596 コドンにおける一塩基置換にて生ずるミスセンス変異体が AT および TM による抗凝固作用に及ぼす影響を評価した。本研究で解析したすべての Arg596 ミスセンス変異型プロトロンビン（596Gln、596Trp、596Gly）は、AT 抵抗性ならびに TM 抵抗性を示した。生体内では、各変異型プロトロンビンの凝固能に依存して血栓症の引き起こしやすさにつながることが推察された。

主 論 文

Abstract

Antithrombin (AT) and thrombomodulin (TM) play important roles in the process of natural anticoagulation *in vivo*. Recently, we reported that the prothrombin Yukuhashi mutation (p.Arg596Leu) was associated with AT and TM resistance-related thrombophilia. To assess the AT and TM resistances associated with other missense mutations by single base substitution in the Arg596 codon, we generated recombinant variants (596Gln, 596Trp, 596Gly, and 596Pro) and investigated the effects on AT and TM anticoagulant functions. All variants except 596Pro were secreted in amounts comparable to that of the wild-type but exhibited variable procoagulant activities. After a 30-min inactivation by AT, the relative residual activity of wild-type thrombin decreased to 15 ± 4.0%, in contrast to values of all variants were maintained at above 80%. The thrombin–AT complex formation, as determined by enzyme-linked immunosorbent assay, was reduced with all tested variants in the presence and absence of heparin. In the presence of soluble TM (sTM), the relative fibrinogen clotting activity of wild-type thrombin decreased to 16 ± 0.12%, whereas that of tested variants was 37%–56%. In a surface plasmon resonance assay, missense Arg596 mutations reduced thrombin–TM affinity to an extent similar to the reduction of fibrinogen clotting inhibition. In the presence of sTM or cultured endothelial-like cells, APC generation was enhanced differently by variant thrombins in a thrombin–TM affinity-dependent manner. These data indicate that prothrombin Arg596 missense mutations lead to AT and TM resistance in the variant thrombins and suggest that prothrombin Arg596 is important for AT- and TM-mediated anticoagulation.

Introduction

Hemostatic disequilibrium is a key mechanism associated with all types of thrombosis. Venous thromboembolism (VTE) is a complex and multifactorial disease involving interactions among acquired factors, such as aging, obesity, surgery, pregnancy, post-partum status, oral contraceptives, and/or cancer, and has also been associated with many congenital risk factors (1). Genetic studies of hereditary thrombophilia have revealed two types of genetic defects, including loss-of-function mutations in natural anticoagulants, such as antithrombin (AT), protein C (PC), and protein S (PS), and gain-of-function mutations in procoagulant factor V (factor V Leiden) and factor II (prothrombin G20210A) (2-4). Among Caucasians, gain-of-function mutations in procoagulant proteins are more prevalent than defects in anticoagulant factors, whereas such mutations are not causative in Asian populations (5).

Thrombin plays central roles in various steps of the blood coagulation process, including the conversion of fibrinogen to fibrin and the activation of platelets and blood coagulation proteins, such as factors V, VIII, XI, and XIII (6). AT is a plasma serine protease inhibitor that inhibits thrombin and other activated serine proteases of the coagulation system. The inhibition of thrombin by AT via the formation of a covalent complex in a 1:1 molar ratio occurs relatively slowly but is dramatically enhanced in the presence of the glycosaminoglycan heparin. Furthermore, the formation of thrombin complexes with the endothelial cell surface receptor thrombomodulin (TM) impairs procoagulant activity and activates PC; activated PC (APC) and PS subsequently inactivate coagulation factors VIIIa and Va.

Recently, we identified a gain-of-function mutation associated with thrombophilia in the gene encoding prothrombin; this mutation, prothrombin Yukuhashi (c.1787G→T, p.Arg596Leu), conveys AT resistance (7). Compared with the wild-type, the mutant thrombin, active form of the prothrombin Yukuhashi, exhibits moderately reduced clotting activity but substantially impaired complex formation with AT. In other words, this mutant thrombin may be resistant to inactivation by AT, thus allowing prolonged procoagulant activity *in vivo* and conferring susceptibility to thrombosis. Subsequently, *F2* c.1787G>A (p.Arg596Gln: prothrombin Belgrade) and *F2* c.1786C>T (p.Arg596Trp: prothrombin Padua2) were

reported as AT resistance mutations in Serbian and Italian thrombosis families, respectively (8, 9). Yukuhashi mutation may also attenuate the inhibitory effect of TM in the thrombin-mediated conversion of fibrinogen to fibrin (i.e., TM resistance), thus possibly contributing to its susceptibility to thrombosis (10).

Crystal structure analysis of the thrombin−AT complex revealed that the side chain of thrombin Arg596 forms two hydrogen bonds with that of AT Asn265 (11). Therefore, Arg596 may be a crucial amino acid for AT-mediated thrombin inactivation. In addition, Arg596 is located in the sodium-binding domain of thrombin, and sodium-bound thrombin (known as the fast form) has been reported to exhibit optimal procoagulant function with increasing substrate specificity for fibrinogen, whereas sodium-free thrombin (slow form) exhibits increasing anticoagulant specificity for TM binding and PC cleavage (12). Therefore, Arg596 may also play important roles in the anticoagulation function of TM.

As single base substitution missense mutations may occur in the human genome at the prothrombin amino acid 596 codon (CGG), we investigated the effects of the single nucleotide substitution prothrombin missense mutations 596Leu (C<u>T</u>G), 596Gln (C<u>A</u>G), 596Trp (<u>T</u>GG), 596Gly (<u>G</u>GG), and 596Pro (C<u>C</u>G) on the anticoagulation functions of AT and TM in this study.

Materials and Methods

Materials

Purified human normal prothrombin, human PC, bovine factor Va, and bovine factor Xa were obtained from Haematologic Technologies (Essex Junction, VT, USA). Human fibrinogen was purchased from Wako Pure Chemical Industries (Osaka, Japan), anti-prothrombin monoclonal antibody was obtained from Abnova Co. (Taipei, Taiwan), and anti-β actin antibody was purchased from Bio Vision (San Francisco, CA, USA). The proteasome inhibitor MG-132 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Neoplastin plus and PTT-Reagent RD were purchased from Roche Diagnostics KK (Tokyo, Japan). *Oxyuranus scutellatus* (Ox) venom (Taipan venom), which was used as a prothrombin activator, was purchased from Latoxan S.A.S (Valence, France). Heparin (unfractionated) was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). Human AT and recombinant soluble thrombomodulin (sTM; Recomodulin®) were generously provided by Mitsubishi Tanabe Pharma Co. (Osaka, Japan) and Asahi Kasei Pharma Co. (Tokyo, Japan), respectively. Sensor chip CM5 and Amine coupling kits containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine hydrochloride-NaOH were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). The selective thrombin inhibitor Pefabloc-TH (NAPAP) was purchased from Pentapharm Ltd. (Basel, Switzerland). The synthetic chromogenic substrates H-D-Phe-Pip-Arg-p-nitroanilide (S-2238) and Glu-Pro-Arg-p-nitroanilide (S-2366) were obtained from Sekisui Medical Co. (Tokyo, Japan).

Recombinant prothrombins

Stable transfectants of human embryonic kidney cells (HEK293) expressing recombinant prothrombins were established as previously described (7). In brief, variant prothrombin expression vectors were prepared to produce amino acid substitutions at Arg596 (CGG) by single base substitution (Leu, CTG; Gln, CAG; Pro, CCG; Trp, TGG; Gly, GGG) using overlap extension polymerase chain reaction (PCR) (13) with respective primer sets to introduce base substitutions. HEK293 cells were transfected with these

variant prothrombin expression vectors using the calcium phosphate method (14). Stable transfectants were subsequently established by G418 selection, and prothrombin expression was determined using dot-blot immunoassays. Stable transfectants were cultured for 24 h in serum-free medium containing 5-μg/mL vitamin K1 (Isei, Yamagata, Japan). Cultured media were collected, centrifuged at 2,000 x g for 10 min to remove cellular debris, concentrated using Vivaspin Turbo 15 (Sartorius Stedim Biotech GmbH, Goettingen, Germany), and stored at −80°C until use. For the thrombin generation assay (TGA), we prepared highly concentrated recombinant prothrombins (1000~ ng/mL) from serum-free conditioned media simply by multiple ultra-filtration to reconstitute the plasma by adding 1/10 volume of them into prothrombin deficient plasma. Prothrombin antigen levels were determined using enzyme-linked immunosorbent assays (ELISAs; Enzyme Research Laboratories, South Bend, IN, USA).

Western blotting analyses

Western blotting was performed according to previously described methods (15). In brief, samples of culture media and cell lysates were separated on 10% SDS-PAGE gels and subsequently transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Protein concentrations were measured using Bio-Rad Protein Assay kits (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and sample volumes were adjusted to standardize quantities of loaded proteins. Membranes were blocked in a solution of 3% dry milk (DM) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (DM-PBS-T) at room temperature for 1 h. An anti-prothrombin mouse monoclonal antibody in DM-PBS-T (1:1000 dilution) was applied to membranes overnight at 4°C, followed by incubation with an anti-mouse IgG HRP-linked antibody in DM-PBS-T (1:2000). As a control, membranes were incubated with an anti-β actin rabbit antibody (1:5000), followed by incubation with an anti-rabbit IgG HRP-linked antibody (1:2000). Signals were visualized using the Immobilon-Western Chemiluminescent HRP Substrate (Millipore). We also performed 10% native-PAGE for cell culture media containing 10 ng recombinant prothrombins followed by Western blotting as described above. As a PIVKA-II positive control, we used cell culture media with

warfarin (1 or 0.1 μg/mL: Wako Pure Chemical Industries) treatment for 48 h, in which the recombinant wild-type prothrombin was secreted.

Procoagulant functions of recombinant prothrombins

To examine the functions of recombinant prothrombins, reconstituted plasma was prepared by mixing prothrombin-deficient plasma (prothrombin activity, <1%; LSI Medience Corporation, Tokyo, Japan) with recombinant prothrombins; a prothrombin plasma concentration of 100 μg/mL was assumed normal (100%). Subsequently, one-stage clotting assays and chromogenic assays using the thrombin specific substrate S-2238 were performed as previously described (16). In brief, reconstituted plasma was diluted 1:100 in buffer (50 mmol/L of Tris-HCl, 0.3 mol/L of NaCl, pH 8.1), and incubated with Ox snake venom (150 μg/mL), phospholipid (50%) and $CaCl_2$ (12.5 mmol/L) for 2 min at 37°C. Then, thrombin activity was determined using an S-2238 by measuring changes in absorbance/min (ΔA/min) at 405 nm with TBA-180 (Toshiba Medical Systems Co, Tokyo, Japan). The 100% phospholipid solution comprised PTT-Reagent RD dissolved in 2 mL of distilled water.

Thrombin inactivation by AT

Inactivation profiles of thrombins derived from recombinant prothrombins were analyzed in the presence of AT as described previously (17). In brief, Ox venom was used to activate recombinant wild-type and variant prothrombins (0.5 μg) to thrombins, which were then inactivated with human AT in the absence of heparin for various time periods. Subsequently, the amidolytic activities of the samples were measured to determine residual thrombin activities as described previously (7). We also performed ELISA to determine the thrombin—AT (TAT) complex formation profiles of recombinant thrombins as described previously (7).

Thrombin generation assay

Wild-type and variant reconstituted plasmas were prepared by mixing prothrombin-deficient plasma with recombinant prothrombins to a final prothrombin concentration of 100% in normal plasma. Normal pooled plasma samples were used as a control, and calibrated automated thrombography (CAT; Thrombinoscope BV, BC Maastricht, The Netherlands) was used to perform TGAs in accordance with the manufacturer's instructions. Reactions were monitored at excitation and emission wavelengths of 390 nm and 460 nm, respectively, for 70 min using a Fluoroscan Ascent FL (Thermo Fisher Scientific KK, Yokohama, Japan) and Thrombinoscope software (Thrombinoscope BV).

Fibrinogen-clotting inhibition by TM

Fibrinogen-clotting assays were performed with or without sTM as described previously (10). Ox venom with phospholipid and $CaCl_2$ was used to convert recombinant prothrombins to thrombins; these were mixed and incubated with sTM solutions at final concentrations of 0, 10, and 25 μg/mL for 1 min to inhibit thrombin activity. Fibrinogen was subsequently added, and clotting times were measured. Relative residual thrombin activity was determined using a thrombin standard curve generated from purified human prothrombin.

Measurement of thrombin binding affinity for TM via surface plasmon resonance

Recombinant variant prothrombins were concentrated to >1,000 μg/mL and diluted to 300 μg/mL in running buffer containing 10 mmol/L Hepes (pH 7.4) and various concentrations of NaCl (0, 150, or 300 mmol/L). Subsequently, 100-μL aliquots were incubated with 200 μL of prothrombin activator solution containing 2.5 μg/mL Ox venom, 10 mmol/L $CaCl_2$, and 5% phospholipid (PTT Reagent RD) at 37°C for 8 min to allow sufficient conversion to thrombin.

Surface plasmon resonance (SPR) experiments were performed using a Biacore3000 device (GE Healthcare Bio-Sciences). sTM in 10-mmol/L acetate buffer (pH 4.0) was initially linked to a CM5 chip

via EDC/NHS coupling, according to the immobilization protocol recommended by the manufacturer. Thrombin solutions were injected in running buffer over the sensor chip surface at a flow rate of 20 μL/min for 5 min, and sensorgrams were collected for thrombin concentrations of 5.26 nmol/L in buffer at 37°C. Analytes were dissociated by replacing the analyte buffer with analyte-free buffer. Nonspecific binding to uncoated flow cells was subtracted from the signals, and dissociation constants (Kds) were calculated by fitting the data from eight injections (0.5, 1.35, 2.65, 5.35, 13.25, 26.35, 52.65, and 132 nmol/L) of thrombin using non-linear regression with a one-to-one Langmuir binding model using BIAevaluation software version 4.4.1.

APC generation assay

Ox venom was used to convert recombinant prothrombins to thrombins; these were subsequently incubated with human PC for 1 h in the presence or absence of sTM. Subsequently, APC activities in samples were determined as described previously (10). We also performed a cell-based APC generation assay using EAhy926 cells, which express TM and endothelial PC receptor (EPCR). We confirmed TM and EPCR expression in these cells by RT-PCR and flow cytometry (data not shown). EAhy926 cells (5×10^5 cells/well in 6-well plates) were cultured for 24 h in Dulbecco's Modified Eagle's Medium with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. The cells were washed 3 times with PBS and incubated with each recombinant prothrombin-derived thrombin (2 μg/well) and purified human PC (16 μg/well) for 3 h in reaction buffer (1 mL) containing 50-mmol/L Tris-HCl (pH 7.5), 100-mmol/L NaCl, and 0.1% BSA. APC generated by variant thrombins was detected in the supernatants by S-2366 cleavage, as described previously (10). To prevent nonspecific S-2366 cleavage by thrombin, Pefabloc-TH was added to each supernatant at 37°C for 30 sec before testing for APC.

Results

Western blotting analysis of recombinant prothrombins in stable transfectants

To examine whether prothrombin proteins were expressed in cells that were stably transfected with recombinant prothrombin expression vectors, culture media and cell lysates were subjected to Western blotting analyses. Prothrombins were sufficiently detectable in culture media and lysates from stable transfectants expressing wild-type, 596Leu, 596Gln, 596Trp, and 596Gly prothrombins, but not from those expressing the 596Pro variant (Fig. 1A, 1B). To validate gamma-carboxylation of the recombinant prothrombins, we carried out a native-PAGE followed by Western blotting and analyzed mobility differences of the secreted prothrombins depending on amounts of negatively charged gamma-carboxyglutamic acids. Without warfarin treatment, the mobility shifts of recombinant wild-type and all tested 596 variant prothrombins were almost same, whereas the significantly slow shifted bands were observed in the recombinant wild-type prothrombins cultured with warfarin (Fig. 1C). These data indicated that majority of the secreted recombinant prothrombins into culture media would have appropriate amounts of gamma-carboxyglutamic acids in the N-terminal region. Lysates of cells stably expressing wild-type and 596Pro variant prothrombins in the presence or absence of 10 μmol/L MG-132, a proteasome inhibitor, were subjected to Western blotting analyses (Fig. 1D). An increased intracellular prothrombin level was observed in stable 596Pro variant cells after treatment with MG-132, whereas the intracellular level of wild-type prothrombin was not affected by the presence or absence of MG-132. Subsequent experiments were performed using all recombinant prothrombins except for 596Pro.

Procoagulant functions of recombinant prothrombins

The procoagulant activities of wild-type and variant recombinant prothrombins in reconstituted plasmas were determined using two methods after mixing prothrombin-deficient plasma with recombinant prothrombins to normal levels, as described in the Materials and Methods. All variant prothrombins exhibited varied but low activities in one-stage clotting assays, whereas comparatively higher activities

were observed in two-stage chromogenic assays. In a subsequent two-stage chromogenic assay, the clotting efficiency of wild-type prothrombin was 97 ± 4.9% (n = 3, mean ± standard error [SE]) of that in normal pooled plasma, whereas the corresponding efficiencies of 596Leu, 596Gln, 596Trp, and 596Gly were 74 ± 5.2%, 116 ± 9.9%, 79 ± 11.2%, and 46 ± 5.4%, respectively (Table. 1).

Thrombin inactivation by AT

An AT resistance analysis of recombinant wild-type and variant thrombins was performed by assessing inactivation profiles in the presence of AT without heparin. After a 30-min inactivation by AT, the relative residual activities of wild-type, 596Leu, 596Gln, 596Trp, and 596Gly recombinant thrombins were 15 ± 4.0%, 96 ± 1.8%, 83 ± 4.2%, 88 ± 4.5%, and 99 ± 0.6%, respectively (Fig. 2; n = 3, mean ± SE). Subsequently, the TAT complex formation profiles of wild-type and variant thrombins were determined using ELISA. In the absence of heparin, TAT formation with wild-type thrombin increased in a time-dependent manner, whereas formation with the variants was near-negligible for the first 30 min (Fig. 3A). After a 60-min incubation, the concentration of TAT complex formed with wild-type thrombin was 2.03 ± 0.15 ng/mL, in contrast to concentrations of 0.07 ± 0.00, 0.16 ± 0.00, 0.03 ± 0.00, and 0.00 ± 0.00 ng/mL with 596Leu, 596Gln, 596Trp, and 596Gly, respectively (n = 3, mean ± SE). The presence of heparin greatly enhanced the formation of TAT complexes with all thrombins, although this process remained substantially impaired with all variants, compared with the wild-type (Fig. 3B). After a 5-min incubation in the presence of heparin, the concentrations of TAT complexes formed with wild-type, 596Leu, 596Gln, 596Trp, and 596Gly thrombins were 2.44 ± 0.006, 1.61 ± 0.13, 1.64 ± 0.16, 0.85 ± 0.14, and 0.38 ± 0.18 ng/mL, respectively (n = 3, mean ± SE).

Thrombin generation assay

Reconstituted plasmas mixed with individual recombinant prothrombins in prothrombin-deficient plasma were compared with normal pooled plasma in TGAs (Fig. 4). The endogenous thrombin potential (ETP),

which corresponds to total thrombin activity, maximum concentration of thrombin (Peak), and duration of thrombin generation (StartTail) are shown in Table 2. The respective values for wild-type reconstituted plasma were similar to those for normal pooled plasma, whereas all variants exhibited a StartTail value more than 2-fold longer than that of wild-type thrombin. These data suggest that the inactivation of all tested variant prothrombin-derived thrombins was very slow when compared to that of wild-type thrombin, thus prolonging procoagulant activity. The peaks of the 596Leu and 596Gln variants were decreased, whereas the corresponding ETPs were increased. However, the 596Trp variant had a very low Peak and decreased ETP, and the 596Gly variant had a low Peak and a similar ETP value to that of the wild-type.

Inhibition of fibrinogen-clotting activity by TM

To examine the effects of TM on the functions of recombinant variant thrombins, fibrinogen-clotting activities were determined in the presence or absence of sTM. The fibrinogen-clotting activity of wild-type thrombin was reduced by sTM in a concentration-dependent manner, with residual activity ratios of 35 ± 0.91% and 16 ± 0.12% in the presence of 10 and 25 μg/mL sTM, respectively (n = 5, mean ± SE; Fig. 5). In contrast, the residual activity ratios of the 596Gln variant decreased from 54 ± 0.54% in the presence of 10 μg/mL sTM to 37 ± 0.48% with 25 μg/mL sTM (n = 5, mean ± SE); this ratio differed significantly from that of wild-type thrombin (Student's *t*-test, $p < 0.001$). The residual activity ratios of the other variants were greater than those of 596Gln in the presence of 10 and 25 μg/mL sTM.

Binding affinity of thrombin for TM

The TM-binding affinities of variant thrombins were evaluated using SPR. Representative sensorgrams obtained in the presence of 150 mmol/L NaCl are shown in Fig. 6A. Missense mutations at Arg596 reduced the thrombin–TM affinity in a similar manner to reductions in fibrinogen clotting inhibition. As shown in Table 3, the 596Gly variant exhibited the lowest affinity for TM (apparent Kd, $7.1 \pm 0.6 \times 10^{-8}$ M), whereas normal thrombin had the highest affinity (apparent Kd, $1.5 \pm 0.1 \times 10^{-8}$ M). Fig. 6B presents

the average binding response (RU) values in the presence of 50, 150, or 300 mmol/L NaCl. The average RU values of wild-type, 596Leu, 596Gln, 596Trp, and 596Gly thrombins in the presence of 150 mmol/L NaCl were 50.4 ± 1.7, 45.4 ± 4.1, 42.6 ± 0.1, 28.8 ± 0.2, and 22.9 ± 0.1; the corresponding values under low NaCl conditions (50 mmol/L) were 158.5 ± 17.2, 192.0 ± 11.5, 65.4 ± 18.0, 143.3 ± 15.9, and 104.5 ± 2.5, respectively, whereas those under high NaCl conditions (300 mmol/L) were 14.1 ± 1.0, 6.1 ± 2.6, 4.6 ± 1.3, 5.3 ± 0.4 and 3.1 ± 0.1, respectively. All tested thrombins exhibited TM affinities at a lower concentration of NaCl (50 mmol/L), whereas only the 596Gln variant exhibited a reduced enhancement of TM affinity.

APC generation assay

APC generated by recombinant thrombin in the presence of sTM was expressed in units of ΔA/min at 405 nm as the difference from the concentration in the absence of sTM. These differences were 0.090 ± 0.002, 0.050 ± 0.001, 0.070 ± 0.002, 0.047 ± 0.001, and 0.017 ± 0.000 for wild-type, 596Leu, 596Gln, 596Trp, and 596Gly thrombins, respectively (Fig. 7A; n = 3, mean ± SE). Very low levels of APC were generated by recombinant thrombins in the absence of sTM (<0.006 ± 0.000, n = 3). A linear relationship between APC activity and 0–0.2 ΔA/min at 405 nm was confirmed in an assay with human APC standards (data not shown). In a 3-h cell-based reaction, APC generated by recombinant thrombin in EAhy926 cells was expressed culture media in units of ΔA/min at 405 nm. These values were 0.055 ± 0.003, 0.033 ± 0.004, 0.038 ± 0.003, 0.026 ± 0.002, and 0.022 ± 0.003 for wild-type, 596Leu, 596Gln, 596Trp, and 596Gly thrombins, respectively (Fig. 7B; n = 3, mean ± SE). The amount of APC generated by wild-type recombinant thrombin in culture media without EAhy926 cells was also very low (0.001 ± 0.000, n = 2).

Discussion

The prothrombin Yukuhashi (c.1787G>T, p.Arg596Leu) mutation has been reported to cause a dysprothrombin that leads to AT and TM resistances causing susceptibility to thrombosis (7, 10). In this study, we investigated the effects of five prothrombin single base substitution missense mutations at Arg596 (596Leu, 596Gln, 596Trp, 596Gly, and 596Pro) on the anticoagulant functions of AT, TM, and PC. Among the tested variant prothrombins at Arg596, the secretion of 596Pro prothrombin was severely impaired. The effects of an experiment with the proteasome inhibitor MG-132 suggest that this protein is subjected to intracellular degradation by the proteasome, as indicated by its poor stability. Therefore, a homozygous *F2* c.1787G>C mutation resulting in the 596Pro variant would likely cause a bleeding tendency similar to those observed in patients with prothrombin deficiencies (18-20).

All of the tested variant thrombins exhibited AT resistance and severely impaired TAT complex formation, suggesting that prothrombin Arg596 is essential for the inactivation of its active form by AT. The TGA is a comprehensive coagulation-function test that allows us to evaluate the initial phase of thrombin generation and the late phase of thrombin inactivation in plasma. In the TGA, all variant prothrombins yielded gentle inactivation phase slopes in the presence of plasma-derived AT, suggesting that the variant thrombins were not inactivated sufficiently by plasma AT and continued to facilitate blood coagulation, despite low levels of procoagulant activity *in vitro*. For TGA in this study, highly concentrated recombinant prothrombins were required to reconstitute the plasma by adding 1/10 volume into prothrombin deficient plasma. We prepared them by multiple ultra-filtrations, which might have damaged the recombinant prothrombins resulting in a reduced thrombin generation peak observed in TGA of 596Trp, even though its delayed StartTail was similar to those of the other variants. In addition, small fluorogenic substrate (Z-GGR-AMC) in TGA may easily access to any variants of thrombin compared with large substrate (fibrinogen), possibly accounting for the lack of agreement between procoagulant activities in the fluorogenic assay (TGA) and in the one-stage clotting assay. Small synthetic substrate (S-2238) was also used in the two stage chromogenic assay. Interestingly, Pechik et al. reported the crystal structure of thrombin in complex with E

domain fragment of fibrin via exosite I, which located away from the active center of thrombin (21). Taken together, all Arg596 mutations, except for 596Pro, may cause AT resistance and susceptibility to thrombosis. Before completed this study, the prothrombin p.Arg596Gln (c.1787G>A) mutation was reported in two unrelated Serbian families with recurrent thrombosis (8). In agreement, plasma samples from these patients indicated apparent AT resistance, and the residual thrombin activity ratios after inactivation by AT were significantly higher than that in plasma from normal individuals.

In addition to AT, TM plays an important role in anticoagulation *in vivo*. Most thrombins, at least those in intact microcirculation, can be rapidly captured by TM on endothelial surfaces to prevent fibrin formation and promote the activation of PC to APC. Accordingly, we analyzed the effects of the Arg596 mutations on the anticoagulant effects of TM using sTM (Recomodulin®) and showed that all variant thrombins were TM-resistant in terms of fibrinogen clotting inhibition. In SPR-based assays and cell-based APC generation assays on EAhy926 cells, we showed that TM enhanced the APC generation activity of all variant thrombins tested, a process that may be reduced in a protease activity- and/or TM binding affinity-dependent manner. These data suggest that, although a lack of inhibition by AT could be of principal importance in a patient with thrombosis, TM resistance may also contribute to thrombotic events to some extent.

Thrombin has been characterized as an allosteric enzyme that is controlled by sodium binding (22, 23), and Arg596 (Arg221a in the chymotrypsinogen numbering system (24)) is located in the sodium-binding region. Sodium-bound thrombin (known as the fast form) has been reported to exhibit optimal procoagulant function with increasing substrate specificity for fibrinogen, whereas sodium-free thrombin (known as the slow form) exhibits increasing anticoagulant specificity for TM binding and PC cleavage (12). Because Arg596 is present in the sodium-binding region of thrombin, mutation at this location is likely to affect sodium binding, and thereby protease activity and specificity. In the SPR assay conducted in this study, a high NaCl concentration (300 mmol/L) attenuated the thrombin–TM affinities of all variants, whereas a low NaCl concentration (50 mmol/L) elevated the affinities of all except the 596Gln

variant. Fuentes-Prior et al. reported the crystal structure of thrombin in complex with the minimal cofactor fragment of TM (EGF456) via exosite I, which located away from the Na+ binding domain of thrombin (25). We do not know the precise mechanisms of action; however, Leu, Trp, Gly and Pro are hydrophobic amino acids, whereas Gln is the only hydrophilic amino acid, which might contribute to being uniquely resistant to allosteric effects of low NaCl condition on TM affinity to the 596Gln variant. This result is attributed to an increase and decrease in the slow form under low and high NaCl conditions, respectively. These results suggest that missense mutations at Arg596 differently influence thrombin–TM affinity.

In the present study, we evaluated the effects of variant thrombins on anticoagulation systems involving AT and TM. A single base substitution missense mutation at amino acid 596 of prothrombin may occur in the human genome, and the associated CpG dinucleotide is a mutation hotspot that is vulnerable to transition from methylcytosine to thymine (26). Accordingly, Cooper and Youssoufian estimated that CpG dinucleotides are up to 42 times more mutable than other sequences in humans (27). Hence, because the wild-type codon 596 (CGG) of prothrombin contains a CpG dinucleotide, the frequency of point mutation at this location may be increased, leading to the possibility of c.1786C>T (p.Arg596Trp: TGG) and c.1787G>A (p.Arg596Gln: CAG) mutations. The p.Arg596Gln mutation was previously reported in two unrelated Serbian thrombophilia families as prothrombin Belgrade (8), in an Indian thrombosis patient as prothrombin Amirta (28), and recently in a Japanese thrombosis family (29). Moreover, c.1786C>T (p.Arg596Trp: TGG) mutation was also reported in two unrelated Italian thrombosis families as prothrombin Padua2 (~~27~~9). We previously demonstrated that the prothrombin Yukuhashi (p.Arg596Leu) mutation resulted in AT resistance in a Japanese thrombophilia family (7). In other words, missense mutations of prothrombin Arg596 have been identified in several thrombosis patients worldwide, suggesting that such mutations may be widely distributed, regardless of race.

In conclusion, we assessed the effects of prothrombin missense mutations caused by single base substitutions at Arg596 on the AT- and TM-dependent anticoagulation systems. The present experiments showed that all variant prothrombins, including 596Leu (prothrombin Yukuhashi), induce AT-resistant and

TM-resistant phenotypes *in vitro*, although the effects of procoagulant activities on thrombosis *in vivo* may vary in a mutation type-dependent manner. Proper association studies are clearly needed to evaluate the association these variants with thrombophilia.

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Figure and table legends

Fig. 1. Western blotting analysis of recombinant prothrombins

Western blotting analyses of cells that stably express recombinant wild-type (WT), 596Leu, 596Pro, 596Gln, 596Trp, and 596Gly prothrombins.

A, Cell culture media except 596Pro; B, Cell lysates; C, Native-PAGE of cell culture media and WT treated with warfarin (1 or 0.1 μg/mL) as a positive controls of dis-gamma carboxyprothrombin; D, Cell lysates from 596Pro variants with or without the protease inhibitor MG-132 (10 μmol/L for 12 h). β-Actin was used as an internal control.

Fig. 2. Profiles of recombinant thrombin inactivation by antithrombin (AT)

Wild-type (WT) and variant thrombins (596Leu, 596Gln, 596Trp, and 596Gly) were derived from respective recombinant prothrombins (1 μg/mL) in diluted reconstituted plasma, and incubated with AT (9.4 μg/mL) for 0–30 min; relative residual amidolytic activities were measured using S-2238. Experiments were performed in triplicate, and data are presented as mean ± standard error (SE). **p< 0.01 (vs. WT: Student's t-test).

Fig. 3. Kinetic analyses of thrombin–antithrombin (TAT) complex formation

A, Kinetic analyses of TAT complex formation in the absence of heparin; B, kinetic analyses of TAT complex formation in the presence of heparin (4 U/mL). Thrombins derived from wild-type (WT) and variant (596Leu, 596Gln, 596Trp, and 596Gly) prothrombins (2 μg/mL) were incubated with antithrombin (AT; 6 μg/mL) for 10, 30, and 60 min in the absence of heparin (A), or 30, 60, and 300 sec in the presence of heparin (B), after which TAT complex concentrations were measured using ELISA. Experiments were performed in triplicate, and data are presented as mean ± SE. *p< 0.05, **p< 0.01 (vs. WT: Student's t-test).

Fig. 4. Thrombin generation assay

Thrombin generation assays (TGAs) were performed using normal plasma and reconstituted plasma samples containing recombinant wild-type (WT) and variant prothrombins in prothrombin-deficient plasma. Calibrated automated thrombography (CAT) was performed using tissue factor (TF) trigger at a final concentration of 5 pmol/L. Experiments were repeated four times, and a representative result is shown.

Fig. 5. Residual ratios of fibrinogen-clotting activity during inhibition by thrombomodulin (TM)

The effects of soluble TM (sTM) on the fibrinogen-clotting activities of recombinant thrombins derived from wild-type (WT) and variant prothrombins (596Leu, 596Gln, 596Trp, and 596Gly) were determined. Samples were incubated with sTM (0, 10, and 25 μg/mL). Experiments were performed in quintuplicate, and data are presented as mean ± SE; *p < 0.001 (vs. WT: Student's *t*-test).

Fig. 6. Thrombomodulin (TM) binding affinities of thrombins in surface plasmon resonance (SPR)-based assays at various concentrations of NaCl

Thrombins derived from normal or recombinant variant prothrombins were incubated with soluble TM (sTM) immobilized on a CM5 sensor chip for 5 min. Three independent experiments were performed.

A, Representative Biacore sensorgrams of the binding of normal and variant thrombins to TM in the presence of 150 mmol/L NaCl are shown. Black, normal; Red, 596Leu; Cyan 596Gln; Orange, 596Trp; Green, 596Gly.

B, Average response (RU) values obtained from sensorgrams of TM-thrombin complex formations in the presence of 50, 150, or 300 mmol/L NaCl are shown. White, 50 mmol/L NaCl; Gray, 150 mmol/L NaCL; Black, 300 mmol/L NaCl.

Fig. 7. Activated protein C (APC) generation assay

A, Thrombins derived from wild-type (WT) and variant recombinant prothrombins were incubated with purified protein C (PC) and soluble thrombomodulin (sTM) for 1 h. B, Thrombins derived from wild-type and variant recombinant prothrombins were incubated with purified PC and EAhy926 cells for 3 h. APC activity was measured using S-2366 and expressed in units of ΔA/min at 405 nm. Data are presented as mean ± SE. Residual thrombin was inhibited by Pefabloc-TH before measuring the activity of generated APC.

Table 1. Relative procoagulant activities of recombinant prothrombins determined by two methods

	One-stage clotting assay	Two-stage chromogenic assay
WT	84 ± 3.0*	97 ± 4.7
596Leu	13 ± 1.3	74 ± 5.2
596Gln	55 ± 3.8	116 ± 9.9
596Trp	12 ± 1.5	79 ± 11.2
596Gly	4 ± 1.5	46 ± 5.4

*, % of normal (n = 3, mean ± SE)

In one-stage clotting assays, the clotting time was measured during the activation of reconstituted plasma containing recombinant wild-type (WT) or variant prothrombins in the presence of tissue factor (TF), calcium, and phospholipid. In chromogenic assays, prothrombins in reconstituted plasmas were sufficiently activated with *Oxyuranus scutellatus* (Ox) venom, and thrombin activities were determined according to amidolytic activity. Normal plasma was assigned a value of 100%. Experiments were performed in triplicate, and data are presented as mean ± SE.

Table 2. Thrombin generation assay

	WT	596Leu	596Gln	596Trp	596Gly	Normal pool
ETP (nM·min)	1256	1545	2321	730	1294	911
Peak (nM)	221	123	204	49	59	194
StartTail (min)	22	56	52	65	62	22

ETP, endogenous thrombin potential; Peak, maximum concentration of thrombin; StartTail, duration of thrombin generation.
Data were derived from Fig. 3.

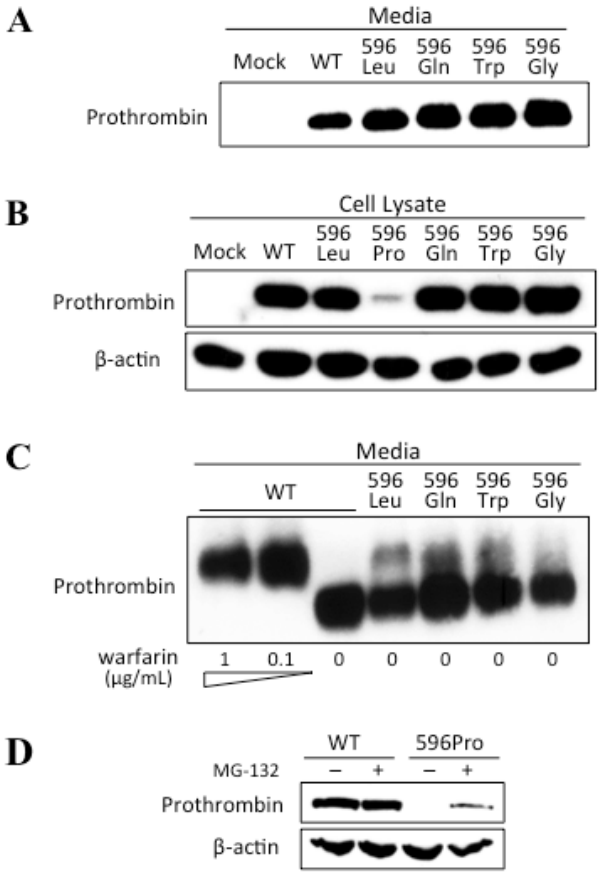
Table 3. Apparent dissociation constants (Kds) for recombinant thrombin binding to TM.

	normal	596Leu	596Gln	596Trp	596Gly
Kd ($\times 10^{-8}$ M)	1.5 ± 0.1*	2.8 ± 0.1	2.0 ± 0.2	3.5 ± 0.2	7.1 ± 0.6

*, mean ± SE (n= 3)

Biacore sensorgrams were collected for thrombin concentrations of 0.5, 1.35, 2.65, 5.35, 13.25, 26.35, 52.65, and 132 nmol/L in buffer at 37ºC. Apparent dissociation constants (Kds) were calculated by fitting the data from eight injections of thrombin using non-linear regression with a one-to-one Langmuir binding model with BIAevaluation software version 4.4.1.

Figure 1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Mock
WT
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596 Gln
596 Trp
596 Gly
Prothrombin
B
Cell Lysate
Mock
WT
596 Leu
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596 Gln
596 Trp
596 Gly
Prothrombin
β-actin
C
Media
WT
596 Leu
596 Gln
596 Trp
596 Gly
Prothrombin
warfarin (μg/mL)
1
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Prothrombin
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Figure 2

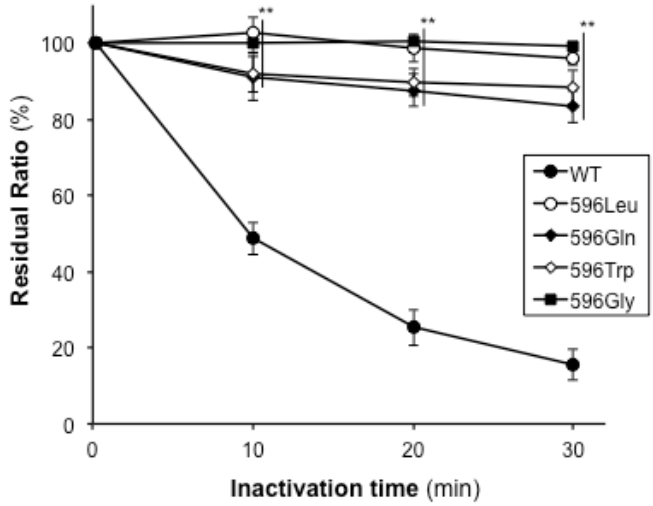

Figure 3

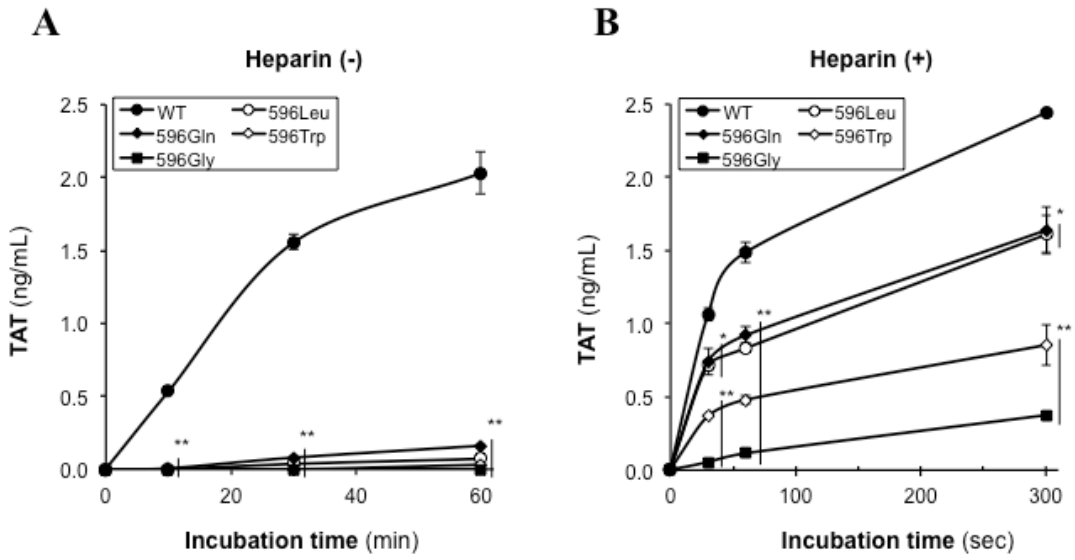

Figure 4

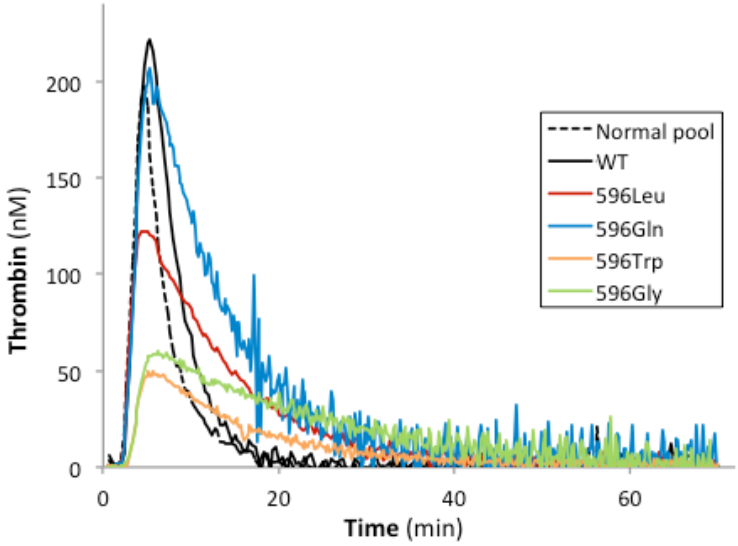

Figure 5

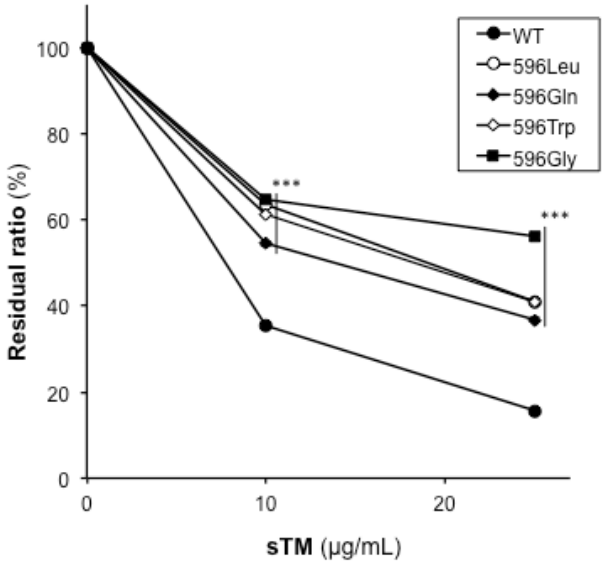

Figure 6

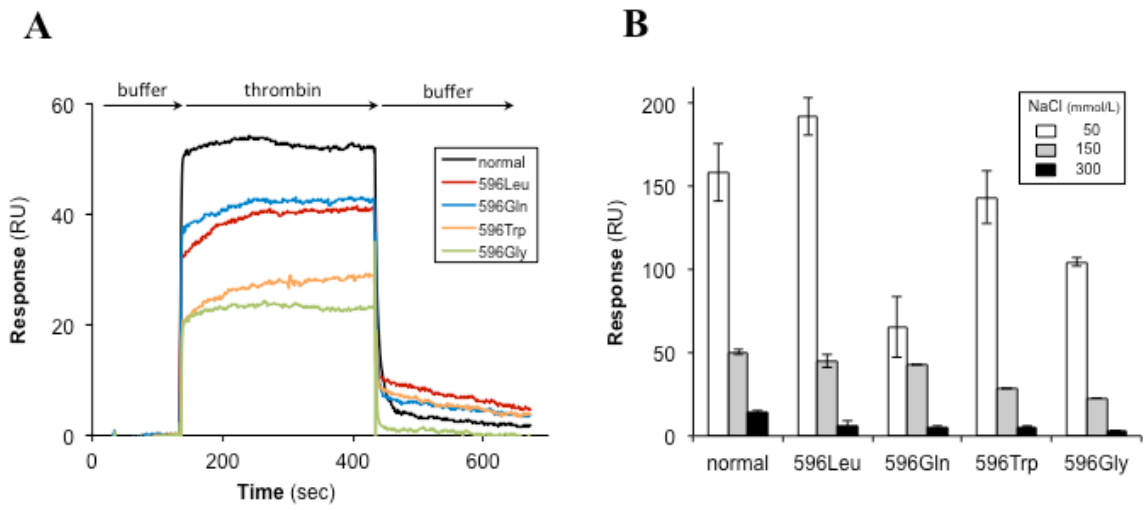

Figure 7