Title: Cartilage Oligomeric Matrix Protein is a Natural Inhibitor of Thrombin

Ying Liang¹, Yi Fu¹, Ruomei Qi², Meili Wang¹, Nan Yang¹, Li He¹, Fang Yu¹, Jian Zhang³,
Cai-Hong Yun⁴, Xian Wang¹, Junling Liu⁵, Wei Kong¹

¹Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking
University; Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing,
P. R. China; ²The Key Laboratory of Geriatrics, Beijing Hospital and Beijing Institute of
Geriatrics, Ministry of Health, Beijing, P. R. China; ³Department of Pathophysiology, Shanghai
Jiao Tong University School of Medicine, Shanghai, P. R. China; ⁴Department of Biophysics,
School of Basic Medical Sciences, Peking University, Beijing, P. R. China; and ⁵Department of
Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment
and Inflammation, Shanghai, P. R. China.

Y. L. and Y. F. contributed equally.

Correspondence: Dr. Wei Kong, Department of Physiology and Pathophysiology, Basic Medical
College of Peking University, Beijing 100191, People’s Republic of China. Tel: +86 10 82805594,
E-mail: kongw@bjmu.edu.cn

Running Title: COMP IS A NATURAL THROMBIN INHIBITOR
**Key Points**

- COMP negatively regulates hemostasis and thrombosis.
- COMP is a natural inhibitor of thrombin.
Abstract

Thrombin is an effector enzyme for hemostasis and thrombosis; however, endogenous regulators of thrombin remain elusive. Cartilage oligomeric matrix protein (COMP), a matricellular protein also known as thrombospondin-5, is essential for maintaining vascular homeostasis. Here, we asked whether COMP is involved in the process of blood coagulation. COMP deficiency shortened tail-bleeding and clotting time and accelerated FeCl₃-induced thrombosis in mice. The absence of COMP had no effect on the platelet count. In contrast, COMP specifically inhibited thrombin-induced platelet aggregation, activation, retraction and the thrombin-mediated cleavage of fibrinogen. Furthermore, surface plasmon resonance (SPR) analysis revealed direct thrombin-COMP binding (K_D=1.38±0.24 μM). In particular, blockage of thrombin exosites with compounds specific for exosite I (hirudin and HD1 aptamer) or exosite II (heparin and HD22 aptamer) impaired the COMP-thrombin interaction, indicating a two-site binding mechanism. Additionally, EGF-like repeats (amino acids 84-261) were identified as a COMP binding site for thrombin. Moreover, COMP was expressed in and secreted by platelets. Using bone marrow transplantation and platelet transfusion to create chimeric mice, platelet-derived but not vessel wall-derived COMP was demonstrated to inhibit coagulation. Taken together, COMP is an endogenous thrombin inhibitor and negative regulator of hemostasis and thrombosis.
Introduction

The serine protease thrombin is the central enzyme in the coagulation cascade. This protein plays a critical role in both hemostasis and thrombosis via the cleavage of fibrinogen to fibrin, the activation of platelets and the conversion of procofactors to active cofactors\(^1\). Thrombin is therefore a viable target for anticoagulation. Exogenous thrombin inhibitors are generally found in the saliva of hematophagous animals. The most characterized inhibitor is hirudin, a 65-residue protein/peptide that binds to thrombin with high affinity\(^2\). In addition, endogenous thrombin inhibitors also exist so that thrombin does not occlude blood flow and cause thrombosis. The current known endogenous thrombin inhibitors, including antithrombin, heparin cofactor II, protein C inhibitor and protease nexin 1, belong to the serpin family\(^3\). Exploring the novel regulatory mechanisms of thrombin will shed light on the prevention and treatment of cardiovascular and thrombus diseases including stroke, atherothrombosis, and deep vein thrombosis.

Cartilage oligomeric matrix protein (COMP), a 524 kDa pentameric noncollagenous glycoprotein, belongs to the thrombospondin (TSP) family and is also known as TSP-5\(^4\). TSPs consist of five homologous members including TSP-1 and -2, which are trimers, and TSP-3, -4 and -5, which are pentamers\(^5\). Recent information regarding the function of TSPs has placed this protein family among the most active and potent regulators of homeostasis and pathologies. For example, TSP-1, the most characterized TSP member, is present in the extracellular matrix, circulates in plasma at low concentrations, and is stored in platelet \(\alpha\) granules where it is released upon activation\(^7\). TSP-1 potentially interacts with several platelet receptors including the integrins \(\alpha\text{v}\beta3\) and \(\alpha\text{IIb}\beta3\), CD36, and CD47 and regulates platelet adhesion, activation and aggregation\(^8\). TSP-2 is required by megakaryocytes for normal platelet formation and function\(^10\). A genetic association study has revealed coronary artery disease and myocardial infarction-associated single-nucleotide polymorphisms in the TSP-1, -2 and -4 genes\(^11\). COMP (TSP-5) is expressed in all types of cartilage, the vitreous of the eye, tendons, heart and vascular smooth muscle cells. Compelling evidence has indicated an essential role for COMP in cartilage/bone metabolism and involvement in arthritis\(^12\). Our recent studies have also revealed that COMP is a central player for
maintaining homeostasis in the cardiovascular system. COMP retains the contractile phenotype of vascular smooth muscle cells and prevents post-injury vascular neointima formation and vascular calcification\textsuperscript{13,14}. COMP deficiency renders spontaneous dilated cardiomyopathy and heart failure in mice\textsuperscript{15}. ADAMTS-7, the only COMP-degrading enzyme identified in vessels\textsuperscript{16}, has been recently reported in a genome-wide study to be significantly associated with coronary artery disease in humans, and it promotes vascular stenosis and calcification in rodents\textsuperscript{17-19}. However, whether COMP affects the coagulation cascade remains unknown. In this study, we explored the potential role of COMP in the regulation of hemostasis and thrombosis.
Methods

Animal Preparation

All studies followed the guidelines of the Animal Care and Use Committee of Peking University. COMP\textsuperscript{-/-} mice in the C57/BL6 background strain were kindly provided by Professor Oldberg Ake from the Department of Cell and Molecular Biology at Lund University, Sweden\textsuperscript{20}. COMP\textsuperscript{+/+} littermates as wild-type (WT) and COMP\textsuperscript{-/-} mice were used for experiments. Animals were genotyped through PCR via respective primers (Supplemental Table I).

Ferric Chloride-induced Carotid Artery Injury

FeCl\textsubscript{3}-induced carotid artery injury was performed as previously described with some modifications\textsuperscript{21}. Briefly, 8-week-old male mice were anesthetized, and the right common carotid artery was exposed. A miniature Doppler flow probe (Model 0.5VB; Transonic Systems, Ithaca, NY) was positioned around the artery, and a 1×2 mm\textsuperscript{2} strip of 1M Whatman filter paper (Whatman International) soaked in 20% FeCl\textsubscript{3} was applied to the adventitia of the artery for 3 min. The filter paper was then removed, and thrombus formation in the artery was monitored via the blood flow rate until complete occlusion (flow rate=0 mL/min).

Radiation Chimeras

Bone marrow transplantation was performed based on previous reports with minor modification\textsuperscript{22,23}. Briefly, mice were exposed to \( \gamma \)-irradiation from a \( ^{60}\text{Co} \) source (Department of Applied Chemistry, Peking University) followed by the injection of bone marrow cells (5×10\textsuperscript{6} cells/mice) via the tail vein. At 4 weeks post-transplantation, tail bleeding time, blood clotting time and FeCl\textsubscript{3}-induced thrombosis of the carotid artery were assessed using the reconstituted mice as described above.

Adoptive Platelets Transfusion

Washed mouse platelets were isolated from WT and COMP\textsuperscript{-/-} mice and then resuspended in the serum of COMP\textsuperscript{-/-} mice at 4×10\textsuperscript{9} platelets/mL. Approximately 0.8×10\textsuperscript{9} platelets (200 \( \mu \)L) were injected into one COMP\textsuperscript{-/-} mouse via the lateral tail vein\textsuperscript{24}. One hour after platelet transfusion, the
mouse tail bleeding time, clotting time and FeCl₃-induced carotid artery injury were measured.

**Statistical Analysis**

Statistical analyses involved the use of GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). All data are presented as the Mean±standard error of the mean (SEM). Comparisons of tail bleeding time, clotting time and FeCl₃-induced thrombosis time in mice are analyzed by nonparametric tests, which are Mann-Whitney test for 2 groups and Kruskal-Wallis test followed by Dunns test for more than 2 groups. The unpaired Student $t$ test (two-sided) is applied for rest comparisons between 2 groups. Comparisons among other greater than 2 groups involved one-way ANOVA followed by the Student-Newman-Keuls test for post-hoc comparison or two-way ANOVA followed by the Bonferroni test. A $P < 0.05$ was considered statistically significant.

**More material and method information in details could be available in Data Supplement.**
Results

COMP−/− Mice Exhibited Enhanced Procoagulant Activity

To investigate the effects of COMP on hemostasis, we first compared the tail bleeding time between COMP−/− and littermate C57/BL6 (WT) mice. Despite normal platelet counts and other hematologic parameters (Supplement Table II), COMP−/− mice displayed significantly shortened tail bleeding time compared with WT mice [Figure 1A; WT vs. COMP−/−: 172±25 s (median 167 s; 95% confidence interval: 154-189.6 s) vs. 114±22 s (median 111 s; 95% confidence interval: 98.69-129.7 s), n=10 for each group, P<0.05]. In addition, the clotting time was markedly reduced in COMP−/− mice compared with WT mice [Figure 1B; WT vs. COMP−/−: 279±47 s (median 270 s; 95% confidence interval: 245.4-312.6 s) vs. 186±28 s (median 180 s; 95% confidence interval: 166.3-205.7 s), P<0.05], indicating that COMP may affect hemostasis. To further investigate the effects of COMP on thrombus formation in vivo, we performed a FeCl₃-induced thrombosis assay using mouse carotid arteries. The time required for vessel occlusion was significantly reduced in COMP−/− mice compared with WT mice (Figure 1C; WT vs. COMP−/−: 7.3±0.8 min vs. 5.0±1.2 min, P<0.05), indicating that COMP is involved in the thrombotic response.

COMP Specifically Targets Thrombin Activity

To further explore the COMP mechanisms affecting procoagulation, we compared the fibrinogen value and coagulation parameters in platelet-free plasma between COMP−/− and WT mice. The fibrinogen concentration in plasma was comparable between WT and COMP−/− mice (Figure 2A; 1.65±0.08 g/L vs. 1.65±0.06 g/L, n=6). We further assessed the plasma coagulation activity using the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) (Figure 2B). There was no significant difference in terms of APTT (WT vs. COMP−/−: 25±0.9 s vs. 24±1.7 s) and PT (WT vs. COMP−/−: 10.6±0.2 s vs. 9.9±0.3 s). However, the
TT value obtained from COMP−/− mice was greatly reduced compared with that from WT mice (WT vs. COMP−/−: 60±2.9 s vs. 43±3.0 s, P<0.05). Furthermore, exogenously purified COMP was further applied in APTT, PT and TT experiments. Intriguingly, COMP dose-dependently (250-1000 ng/mL) prolonged the TT value in the plasma of WT mice, but only increased APTT and PT values at high concentration (1000 ng/mL; Figure 2C). Similar observations were obtained in human plasma (Supplemental Figure I). Together, these data indicate that COMP may affect the thrombin activity on the cleavage of fibrinogen to fibrin.

Another important role of thrombin is to activate platelets. Exogenously purified COMP dose-dependently (100-300 ng/mL) inhibited thrombin (0.1 U/mL)-induced mouse platelet aggregation (Figure 2D). In contrast, COMP exhibited no effect on ADP (10 μM)- or collagen (1 μg/mL)- induced platelet aggregation. The inhibitive effect of COMP on platelet aggregation was also validated in human washed platelets (Supplemental Figure IIa). Concomitantly, COMP specifically prevented thrombin but not ADP or collagen-induced ATP release in mouse (Figure 2E) platelets. Similarly COMP also negatively affected thrombin-induced ATP release in human platelets (Supplemental Figure IIb). Moreover, the thrombin-induced translocation of phosphatidylserine from inner to outer leaflet of the plasma membrane, as a marker of activated platelets detected by FITC-conjugated Annexin V, was repressed by purified COMP (300 ng/mL) (Figure 2F), indicating that COMP inhibited thrombin-induced platelet activation. In contrast, collagen-induced platelet activation was not affected by purified COMP (data not shown). In addition, the rate of thrombin-induced clot retraction in platelet rich plasma from COMP−/− mice was markedly accelerated compared with that of WT mice, whereas exogenous supplementation with purified COMP (300 ng/mL) circumvented the enhanced clot retraction of COMP−/− platelet rich plasma (Figure 2G). Thus, COMP may suppress thrombin-regulated platelet retraction. Together, COMP specifically targeted thrombin-induced platelet aggregation, secretion, activation, and retraction.

The pleiotropic effects of thrombin on cells are mediated by the protease activated receptor (PAR) family of G-protein coupled receptors (GPCR), which includes PAR-1 to 4. Human platelets express PAR-1 and PAR-4, and the cleavage of each receptor initiates signaling cascades.
Mouse platelets express PAR-3 and PAR-4, but PAR-3 does not mediate signaling, making PAR-4 the sole signaling receptor. To explore whether COMP affects thrombin-initiated platelet activation by affecting membrane-bound PAR receptors, the specific PAR-4 activator AYPGKF-NH₂ was used to induce platelet aggregation. However, exogenous COMP at concentrations ranging from 100 to 300 ng/mL exhibited no effect on PAR-4 activator-induced platelet aggregation (Supplemental Figure III). These data indicated that COMP may preferentially target thrombin rather than thrombin receptors. Thus, COMP may serve as endogenous modulator of thrombin.

**SPR examination of the COMP-Thrombin Interaction**

Next, we asked whether COMP directly binds to thrombin. An increasing amount (0.125-8 μM) of recombinant α-thrombin was injected over a CM5 sensor chip immobilized with purified COMP. A dose-dependent increase in SPR signaling was observed after injection, indicating the binding of thrombin to COMP, while reduced signaling, demonstrating the dissociation process, existed once cease of injection (Figure 3A). Notably, thrombin did not digest COMP within the duration of SPR experiments (Supplemental Figure IV), indicating that the data obtained from SPR was from the direct binding of thrombin to full-length COMP rather than a COMP fragment. The 1:1 Langmuir model was applied for data stoichiometry best fitting to yield a $K_D$ value of 1.38 ±0.24 μM for thrombin, implying COMP directly bound to thrombin with moderate affinity (Figure 3B, n=3).

**COMP Binds to Thrombin at Exosite I and II Through Its EGF Domain.**

Thrombin has two distinct electropositive surface domains, termed as exosite I and II, that contribute to the specificity of thrombin for recognizing its substrates, inhibitors and receptors. To define the COMP mechanism for binding to thrombin, we then used selective exosite ligands i.e., the DNA aptamer HD1 and hirudin to block exosite I and the DNA aptamer HD22 and heparin to block exosite II for further SPR analysis. Both hirudin (Figure 3C) and HD1 (Figure 3D) competitively inhibited thrombin binding to immobilized COMP. The corresponding IC₅₀ values
were 455.5±44.1 nM for hirudin and 563.8±64.7 nM for HD1. Similarly, heparin (Figure 3E) and HD22 (Figure 3F) inhibited thrombin binding to immobilized COMP (IC\textsubscript{50} values: 4994±919 nM for heparin and 1461±269 nM for HD22). These finding suggested that COMP bound to thrombin via both exosite I and exosite II. To investigate the binding domain of COMP, we reconstructed maltose binding protein (MBP)-fused COMP domains, including the N-terminus (amino acids: 20-83), the EGF-like repeats (amino acids 84-261), the Type III repeats (amino acids 266-520), and the C-terminus (amino acids 521-755). Only the MBP-fused EGF-like repeats of COMP pulled down purified thrombin, indicating direct binding of the COMP-EGF domain to thrombin (Figure 3G). However, COMP displayed no effects on the peptidase activity of thrombin despite of interaction with exosite I and II, demonstrated by the amidolysis of small peptide substrates S2238 and Fluo-substrates (Figure 3H-I).

**Bone Marrow derived COMP Negatively Regulated Hemostasis and Thrombosis**

To evaluate the cellular origin of the COMP involved in hemostasis and thrombosis in vivo, we generated radiation chimera mice. Bone marrow cells were isolated from WT and COMP\textsuperscript{-/-} mice and injected into lethally irradiated male WT and COMP\textsuperscript{-/-} mice, respectively. To evaluate the degree of engraftment following irradiation, CD45.1\textsuperscript{+} cells in the peripheral blood were assessed by flow cytometry in CD45.2 mice transplanted with CD45.1 bone marrow cells. The percentage of CD45.1\textsuperscript{+} cells in peripheral leucocytes was not significantly different between the WT and COMP\textsuperscript{-/-} mice (70-93%) following bone marrow reconstitution (data not shown). PCR analysis of whole blood derived from chimeric mice further confirmed that the bone marrow from donor mice was reconstituted in recipient mice (Supplemental Figure V). The hematological values of the blood cells were comparable among the four groups of chimeric mice (Supplemental Table III). These results indicated that bone marrow reconstitution and refreshed blood profiles of the WT and COMP\textsuperscript{-/-} recipient mice were equally modulated by irradiation-coupled transplantation. The tail bleeding time of WT mice engrafted with bone marrow from WT mice was 167±27 s (median 167 s; 95% confidence interval: 138.5-195.2 s, n=6), whereas COMP\textsuperscript{-/-} bone marrow chimeras had markedly shortened bleeding time [102±26 s (median 100 s; 95%
confidence interval: 74.6-128.4 s, n=6, P<0.05). In contrast, the bleeding time of COMP<sup>+</sup> mice engrafted with bone marrow from WT mice was similar to that of WT chimera mice [174±40s (median 170 s; 95% confidence interval: 132-216 s, n=6)]. There was no significant difference between WT and COMP<sup>+</sup> mice engrafted with bone marrow from COMP<sup>+</sup> mice (Figure 4A). Similar results were obtained regarding the clotting time (Figure 4B). Moreover, artery thrombosis was induced by FeCl₃ in the four groups of the chimeric mice. In line with the above observations, COMP deficiency in the bone marrow but not in recipient mice accelerated FeCl₃-induced thrombosis in vivo (Figure 4C). To explore whether the affected hemostasis and thrombosis in chimera mice was due to the altered thrombin activity in the presence or absence COMP, TT values were measured in chimeric mice after reconstitution (Figure 4D). Interestingly, COMP deficiency in bone marrow of donor mice greatly shortened the TT time in both WT and COMP<sup>+</sup> recipient mice, whereas COMP deficiency in recipient mice did not significantly affect the TT compared to the respective WT recipient. These data provide a direct evidence that the bone marrow-derived COMP is accountable for the inhibited thrombin activity and procoagulant function in vivo.

**Platelets Secreted COMP**

Platelets are derived from megakaryocytes in the bone marrow; thus, we asked whether COMP is expressed in platelets. Indeed, COMP protein was detected by Western blotting using human, rat and mouse resting platelets (Figure 5A). Co-immunofluorescence staining further revealed the colocalization of COMP with vWF and TSP-1, two recognized α-granule proteins, indicating the existence of COMP in resting platelets from humans (Figure 5B) and mice (Supplemental Figure VI). Furthermore, the COMP in thrombin-activated platelets was assessed. Considering the potential interference of divalent cations such as Ca<sup>2+</sup> or Mg<sup>2+</sup> on the platelet secretion of some glycoprotein such as TSP-1<sup>27</sup>, we measured the COMP expression and secretion by activated platelets in the presence of EDTA. COMP was barely detectable in the supernatant of resting platelets. Notably, thrombin (0.1 U/mL) time-dependently increased COMP in the supernatant of platelets from 1 to 60 min (Figure 5C), in parallel with TSP-1 secretion, indicating
COMP might be released from α-granules of platelets. Moreover, thrombin in the 0.02-0.1 U/mL concentration range dose-dependently stimulated COMP secretion (Figure 5D). In contrast, the COMP level was not significantly affected in the cellular pellet of platelets. Thus, the total COMP of platelets lysates containing both pellet and supernatant was increased following thrombin stimulation (Supplemental Figure VIIa). Interestingly, application of cycloheximide, the inhibitor of protein biosynthesis, reversed the thrombin-elevated COMP in platelets. In contrast, the pretreatment of MG132, the inhibitor of proteasome degradation, had no effect on COMP upregulation (Supplemental Figure VIIb), implying that thrombin-upregulated COMP expression within 1 h was not related to inhibition of COMP degradation. Moreover, there was no significant change of COMP on platelet surface following thrombin stimulation (Supplemental Figure VIIc).

Together, these data indicated that thrombin enhanced COMP level was due to de novo protein synthesis in activated platelets. Moreover, other platelets activators including PAR-4 agonist, collagen and ADP all significantly increased COMP expression in platelets lysates as well (Supplemental Figure VIIId). It implied that the increased COMP was mainly related to the platelet activation, which was further demonstrated by that U73122, a phospholipase C (PLC) inhibitor which inhibited platelet activation, blocked the platelet activation-induced COMP expression (Supplemental Figure VIIe).

To further confirm the bona fide interaction between thrombin and platelet-derived COMP, we performed coimmunoprecipitation assays using the supernatant of thrombin-stimulated platelets (Figure 5E). COMP was only present in thrombin antibody precipitated samples but not in IgG controls. In addition, thrombin was only present in COMP antibody-precipitated samples but not in IgG controls. These data reinforce the ideas that activated platelets upregulate and secrete COMP and platelet-derived COMP is associated with thrombin.

**Platelet-derived COMP Inhibited Thrombin Activity and Procoagulant Function**

Next, we asked whether platelet-derived COMP regulates thrombin activity. To address this question, we first compared platelet aggregation in response to thrombin (0.1 U/mL) between wild type and COMP<sup>−/−</sup> mice. Within 2 min, greater than 70% of the platelets identically aggravated in both mouse groups (Figure 6A). However, pretreatment of the platelets with a low concentration (0.02 U/mL) of thrombin at 37°C for 1 h, which did not cause platelet aggregation (Data not
shown) but significantly upregulated COMP expression and secretion (Figure 5D), led to the reduction of WT platelet aggregation by additional 0.08 U/mL thrombin stimulation compared to COMP<sup>−/−</sup> platelets (Figure 6A; WT vs. COMP<sup>−/−</sup>: 23±4.1% vs. 47±3.0%, P<0.05). In contrast, pretreatment of thrombin (0.02 U/mL, 1 h) on both WT and COMP<sup>−/−</sup> platelets led to the same decrease of PAR-4 agonist-induced platelet aggregation, implying no difference of PAR-4 receptor desensitization between two genotypic platelets [Supplemental Figure VIIIa; WT vs. COMP<sup>−/−</sup>: 64±2.4% / 44±2.4% vs. 62±4.1% / 45±1.9% (without / with pretreatment)]. Furthermore, thrombin-induced TSP-1 release displayed identically in WT and COMP<sup>−/−</sup> platelets (Supplemental Figure VIIIB). These data indicate that platelet-derived COMP negatively regulates thrombin activity in vitro.

To further confirm the role of platelet-derived COMP on procoagulant function in vivo, washed platelets purified from WT and COMP<sup>−/−</sup> mice were reconstituted with COMP<sup>−/−</sup> platelet-free plasma, which was used to exclude the effects of COMP in WT plasma, and then transfused into recipient COMP<sup>−/−</sup> mice. COMP<sup>−/−</sup> recipient mice that received COMP<sup>−/−</sup> donor platelets had a tail bleeding time of 112±22 s (median 106 s; 95% confidence interval: 88.2-135.1 s, n=6). Strikingly, the tail bleeding time was greatly prolonged to 204±39 s (median 205 s; 95% confidence interval: 162.9-244.5 s, n=6) in COMP<sup>−/−</sup> recipient mice receiving WT donor platelets (Figure 6B). A similar finding was observed with regard to clotting time (Figure 6C). Moreover, WT platelet transfusion prolonged FeCl<sub>3</sub>-induced artery thrombus formation in COMP<sup>−/−</sup> mice compared with COMP<sup>−/−</sup> platelets transfusion [Figure 6D; 6.9±1.0 min (median 7.2 min; 95% confidence interval: 5.9-7.9 min) vs. 3.8±0.9 min (median 3.5 min; 95% confidence interval: 2.9-4.8 min), n=6]. Taken together, these results highlight the importance of platelet-derived COMP as a critical negative-feedback regulator in thrombin-related hemostasis and thrombosis.
Our study revealed COMP as a natural thrombin inhibitor and an endogenous anticoagulant protein. The upregulation and release of COMP from platelets upon activation may constitute an important negative-feedback regulation of thrombin-related blood coagulation.

The blood coagulation cascade cannot occur without the action of the serine protease thrombin. Therefore, direct inhibitors of thrombin are now being developed for a promising new generation of anticoagulants for the prevention and treatment of acute coronary artery diseases and thrombotic disorders, providing the advantages of efficacy, safety and tolerability over heparins and vitamin K antagonists. In the search of direct inhibitors of thrombin, a number of compounds have been identified, including those derived from the tripeptide template D-Phe-Pro-Arg, aptamers and peptides isolated from blood-sucking animals\textsuperscript{28}. Moreover, thrombin has non-hemostatic functions attributable to atherosclerosis and atrial fibrillation formation. For example, ApoE\textsuperscript{-/-} mice with genetically reduced levels of thrombin or direct thrombin inhibitor have decreased atherosclerosis, increased plaque stability and a decreased pro-inflammatory profile\textsuperscript{39}. The activity of thrombin is tightly regulated but the regulatory mechanism is yet to be fully understood. Endogenous thrombin inhibitors include antithrombin, heparin cofactor II, protein C inhibitor and protease nexin 1, which belongs to the serpins family. A recent study has also indicated that β2-Glycoprotein I functions as a physiological anticoagulant by inhibiting the activity of thrombin\textsuperscript{30}. Here, we identified COMP as a novel endogenous physiological regulator of thrombin. COMP directly binds to thrombin and specifically inhibits thrombin-induced platelet aggregation, activation, secretion and contraction, as well as the conversion of fibrinogen to fibrin. Moreover, COMP\textsuperscript{-/-} mice exhibited shortened tail bleeding time and accelerated thrombosis.
formation in injured vessels. Thus, by fine-tuning thrombin activity, COMP is not only important for maintaining hemostasis but is also involved in pathological thrombosis.

Thrombin has two anion-binding exosites, exosite I and II, which are essential for its activity and specificity. Some thrombin ligands specifically bind to either exosite I or II, while others engage both exosites. Exosite I is the main binding site for fibrinogen, thrombomodulin, PAR1, factors V and VIII, protein C and FXIII. Exosite II is known as a heparin binding site, and plays a role in platelet binding via GPIbα and the direct recognition of some substrates such as FV and FVIII. Here, we described COMP binding to thrombin at an approximate $K_D$ value of 1.38 μM, which is similar to the binding affinity of PAR-1 and fibrinogen to thrombin. In particular, the blockage of thrombin exosites with compounds specific for exosite I or exosite II impaired the COMP–thrombin interaction, indicating that COMP binds to both exosites. However, COMP could not directly affect the peptidase activity of thrombin. Thus, this moderate COMP binding to thrombin may capture the binding sites for thrombin substrates and prevent excessive thrombin activation during physiological and pathological states.

Recent progress in the platelet transcriptome has revealed that thousands of genes are present in platelets. Recent advances in platelet proteomics has also uncovered hundreds of proteins released from activated platelets with unknown function, which may contribute to atherosclerotic or thrombotic diseases. Here, we identified COMP expression in α-granules from resting platelets released upon activation. Furthermore, bone marrow transplantation and platelet transfusion experiments indicated that platelet-derived COMP negatively regulated the coagulation cascade. Interestingly, TSP-1, one of the most abundant α-granule proteins belonging to the same thrombospondin (TSP) family as COMP, appears to exhibit a different function on
hemostasis and thrombosis. TSP-1 is a trimeric glycoprotein secreted from α-granules from platelets upon their activation. TSP-1 potentially interacts with several platelet receptors including the integrins αvβ3 and αIIbβ3, CD36, and CD47. TSP-1 induces platelet activation likely in an indirect mechanism via CD36-dependent cAMP-PKA signaling pathway inhibition or by blocking the antithrombotic activity of nitric oxide/cGMP signaling. In addition, TSP-1 protects endothelial vWF from cleavage by ADAMTS13, enhancing the dynamic recruitment of platelets into developing thrombi. TSP-1 mice exhibit a prolonged occlusion time upon photochemical injury in venules and arterioles compared with WT vessels. In contrast, COMP is a pentameric glycoprotein that is released from platelets upon activation. COMP specifically inhibits thrombin activity by directly binding to thrombin. COMP neither affects ADP or collagen-induced platelet activation, nor does it interfere with the thrombin receptor. COMP mice demonstrated a shortened tail-bleeding and occlusion time upon vascular injury. These results demonstrate negative feedback regulation of thrombin-induced platelet activation and the coagulation cascade by platelet-derived COMP. The importance of COMP in atherothrombosis and thromboembolic disease needs to be explored further. Targeting COMP-thrombin may shed light on the novel anticoagulant discovery.
Acknowledgement

We are grateful for the technical supports from Dr. Weijuan Yao (PT, APTT, and TT, and the fibrinogen tests) and Prof. Guoqing Liu (mice bone marrow transplantation) in Peking University. Prof. Chang Chen from Institution of Biophysics in Chinese Academy of Sciences is warmly appreciated for providing the support of SPR experiment. We thank Prof. Oldberg Ake from Lund University, Sweden for kindly providing the COMP−/− mice.

This research was supported by funding from the International Cooperation and Exchanges NSFC (81220108004) (W. K.), the National Program on Key Basic Research Projects (973 Program) (2012CB518002) (W. K.), the National Natural Science Foundation of the P.R. China (Nos. 81070243, 81121061, 91339000) (W. K.), the National Science Fund for distinguished Young Scholars (81225002) (W. K.), the “111” Project of Chinese Ministry of Education (No. B07001) and the National Science Fund for Young Scholars (81300198) (Y. F.).
Authorship Contributions

Y. L. and Y. F. equally designed and performed the experiments, analyzed and interpreted the data/results, and wrote the manuscript.

R. Q. performed platelet aggregation experiments.

M. W., L. H. and F. Y. performed mouse blood collection and coagulation experiments.

N. Y. organized and made the figures.

J. Z. and C.-H. Y. provided suggestions for the experimental design.

J. L. and X. W. designed portions of the study and edited the manuscript.

W. K. designed experiments, interpreted data, and wrote and edited the manuscript.

All authors have no conflicts of interest.
References


11. Stenina OI, Topol EJ, Plow EF. Thrombospondins, their polymorphisms, and


36. Bonnefoy A, Moura R, Hoylaerts MF. The evolving role of thrombospondin-1 in


Figure Legends

Figure 1. Hemostasis and thrombosis in WT and COMP<sup>−/−</sup> mice. (A) Tail bleeding time in WT and COMP<sup>−/−</sup> mice. n=10, *P<0.05. (B) Blood cloting time of WT and COMP<sup>−/−</sup> mice. n=10, *P<0.05. (C) Left: Representative Doppler echocardiogram of the blood flow rate in mouse right carotid arteries following FeCl<sub>3</sub> injury. The time when the flow rate was reduced to 0 mL/min was considered as the complete occlusion time. Right: Statistic results of the right carotid artery complete occlusion time. n=10, *P<0.05.

Figure 2. COMP regulates thrombin activity. Serum fibrinogen (FIB, A), activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) (B) of the platelet-free plasma isolated from WT and COMP<sup>−/−</sup> mice (n=6, *P<0.05; NS, no significance). (C) APTT, PT and TT of WT platelet-free plasma in the absence or presence of purified COMP. Platelet-free plasma that had no coagulation at 600 s is shown as >600s. (n=4, *P<0.05 vs. 0 ng/mL COMP) (D) Upper: Representative platelet aggregation tracings following thrombin (0.1 U/mL), collagen (1 μg/mL) or ADP (10 μM) treatment with or without purified COMP. Lower: Statistical results show the maximal percentage of platelet aggregation (n=4, *P<0.05 vs. 0 ng/mL COMP). (E) Measurement of ATP released in the supernatant of platelets treated with thrombin (0.1 U/mL), collagen (1 μg/mL) or ADP (10 μM) in the absence or presence of COMP for 5 min. (n=4, *P<0.05 vs. 0 ng/mL COMP). (F) Representative flow cytometry analysis of phosphatidylserine on the surfaces of platelets treated with thrombin in presence or absence of COMP (300 ng/mL) for 15 min. Bar graph shows statistical results of the mean fluorescence intensity (MFI). n=4, *P<0.05. (G) Left: Representative images of the clots in thrombin-induced
mouse platelet-rich plasma with or without purified COMP (300 ng/mL) at different time points. Right: Statistic bar graph shows the percentage of non-clot size to initial clot size. (n=4, *P<0.05).

**Figure 3. Direct interaction between COMP and thrombin.** (A) Representative surface plasmon resonance analysis of different concentrations of thrombin injected through a biosensor chip conjugated with COMP. The altered response unit (ΔRU) is recorded in real-time after perfusion. (B) The best fitting of plot data in thrombin concentrations and their corresponding ΔRUs yields a $K_D$ of 1.38±0.24 μM for thrombin based on 1:1 Langmuir model. (n=3) Thrombin (1 μM) in presence of hirudin (C), HD1 (D), Heparin (E) and HD22 (F) at different concentrations was injected over a COMP-linked biosensor chip. The percentage of ΔRU to the ΔRU in thrombin (1 μM) injection alone (thrombin bound) is regressed with according concentrations of hirudin, HD1, heparin or HD22 (n=3). Concentrations corresponding to 50% of thrombin bound are the IC50. (G) Western blot of protein fractions precipitated with amylase beads from a mixture of thrombin (3 μg) and different purified MBP-fused COMP protein fragments. Fractions before precipitation were applied as input for loading control. Thrombin (0.1 U/mL)-cleaved chromogenic substrate S2238 (50 μM) (H) and fluorogenic substrate FluCa (2.5 mM) (I) were measured with absorbance at 405 nm and fluorescent intensity (excitation: 390nm; emission: 460nm) respectively. Hirudin (0.1 mg/mL) was applied as positive controls. n=3, *P<0.05 vs. 0 ng/mL.

**Figure 4. Bone marrow (BM) or non-BM-derived COMP in hemostasis and thrombosis.** Chimeric mice were created by WT or COMP−/− (recipients) mice cross-transplanted with bone
marrow from WT or COMP<sup>−/−</sup> mice (donors). Tail bleeding time (A), blood clotting time (B) and carotid artery occlusion time following FeCl<sub>3</sub> injury (C) in chimeric mice. (D) Thrombin time (TT) was measured in the platelet-free plasma isolated from chimeric mice. n=6 for each group, *<sup>P<0.05</sup>; NS, no significance.

**Figure 5. COMP expression and secretion in platelets.** (A) Western blot of suspensions of resting platelets without centrifugation from humans, rats and mice. (B) Representative immunofluorescence images of human resting platelets (Scale Bar, 5 μm). Mouse washed platelets were treated with thrombin (0.1 U/mL) at different time points (C) or stimulated with thrombin for 60 min at different doses (D). Representative Western blot analysis for supernatant and pellet of platelets isolated from platelet suspensions by centrifugation. Protein band density was normalized to the corresponding β-actin and then to the mean of the corresponding control group (0 min or 0 U/mL). Bar graphs show the band densitometry with statistics. n=3, *<sup>P<0.05</sup> vs. 0 min (C) or 0 U/mL (D). (E) Co-immunoprecipitation (IP) of COMP and thrombin in the supernatant of thrombin-activated mouse platelets (0.1 U/mL, 60 min). Rabbit IgG was as negative control for IP. Input fractions isolated prior to precipitation were detected for loading controls.

**Figure 6. Platelet-derived COMP involvement in hemostasis and thrombosis.** (A) Representative platelet aggregation tracings following different thrombin treatments, 0.1 U/mL (Upper) or 0.02 U/mL for 1 h plus additional 0.08 U/mL thrombin (Lower). Bar graph shows statistical results of aggregation maximal percentages from 4 independent results. *<sup>P<0.05</sup>; NS, no significance. Tail bleeding time (B), blood clotting time (C) and carotid artery occlusion time
(D) in COMP$^+$ mice with platelet transfusion. n=6, *$P<0.05$. 
Figure 1
Figure 3
Figure 6
Cartilage oligomeric matrix protein is a natural inhibitor of thrombin

Ying Liang, Yi Fu, Ruomei Qi, Meili Wang, Nan Yang, Li He, Fang Yu, Jian Zhang, Cai-Hong Yun, Xian Wang, Junling Liu and Wei Kong