Thymidine Phosphorylase Participates in Platelet Signaling and Promotes Thrombosis

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Rationale: Platelets contain abundant thymidine phosphorylase (TYMP), which is highly expressed in diseases with high risk of thrombosis, such as atherosclerosis and type II diabetes mellitus.

Objective: To test the hypothesis that TYMP participates in platelet signaling and promotes thrombosis.

Methods and Results: By using a ferric chloride (FeCl₃)-induced carotid artery injury thrombosis model, we found time to blood flow cessation was significantly prolonged in Tymp⁻/⁻ and Tymp⁺/⁻ mice compared with wild-type mice. Bone marrow transplantation and platelet transfusion studies demonstrated that platelet TYMP was responsible for the antithrombotic phenomenon in the TYMP-deficient mice. Collagen-, collagen-related peptide-, adenosine diphosphate-, or thrombin-induced platelet aggregation were significantly attenuated in Tymp⁻/⁻ and Tymp⁺/⁻ platelets, and in wild type or human platelets pretreated with TYMP inhibitor KIN59. Tymp deficiency also significantly decreased agonist-induced P-selectin expression. TYMP contains an N-terminal SH3 domain-binding proline-rich motif and forms a complex with the tyrosine kinases Lyn, Fyn, and Yes in platelets. TYMP-associated Lyn was inactive in resting platelets, and TYMP trapped and diminished active Lyn after collagen stimulation. Tymp/Lyn double haploinsufficiency diminished the antithrombotic phenotype of Tymp⁺/⁻ mice. TYMP deletion or inhibition of TYMP with KIN59 dramatically increased platelet-endothelial cell adhesion molecule 1 tyrosine phosphorylation and diminished collagen-related peptide– or collagen-induced AKT phosphorylation. In vivo administration of KIN59 significantly inhibited FeCl₃-induced carotid artery thrombosis without affecting hemostasis.


Key Words: intracellular signaling ■ platelet ■ thrombosis ■ thymidine phosphorylase

Thrombotic events are a major cause of morbidity and mortality in developed nations and remain an important area for new therapeutic discoveries. Inappropriate or uncontrolled platelet activation at site of vascular injury is an important pathogenic component of thrombosis, which causes myocardial infarction or stroke. Therefore, understanding the complex signaling pathways involved in promoting and inhibiting platelet activity remains high priority.

In This Issue, see p 961

Human platelets contain >4,000 proteins,¹ and for the most part the functions of these proteins are not known with certainty. Thymidine phosphorylase (TYMP) was initially purified from human platelets, and each human platelet contains ≈116,000 copies of TYMP.¹ TYMP reversibly converts thymidine to thymine and 2-deoxy-d-ribose-1-phosphate (2DDR),² and the latter sugar is further degraded to 2-deoxy-d-ribose (2DDR). TYMP thus maintains the nuclear pool of these molecules and contributes to the nucleotide salvage pathway. In 1987, a potent angiogenic protein, platelet-derived endothelial cell growth factor, was purified from human platelets and was later shown to be identical to human TYMP.³ The angiogenic effect of TYMP³ is supported by observations that TYMP expression in tumors is associated with enhanced angiogenesis.⁴,⁵ The angiogenic effect of TYMP is thought to be mediated by its mobilizing effect on endothelial cells, but the actual mechanism(s) remain undefined.⁶,⁷ We have found that direct injection of a plasmid vector encoding human TYMP into ischemic canine myocardium or...
rabbit hindlimb promotes angiogenesis in cardiac and skeletal muscle and improves myocardial function and prevents hindlimb necrosis. We also found that TYMP inhibited vascular smooth muscle cell proliferation and migration. The inhibitory role of TYMP on vascular smooth muscle cell is partly mediated by regulating the phosphorylation of Lyn, an important nonreceptor tyrosine kinase of the Src family. TYMP is also expressed in monocytes/macrophages and stromal cells, suggesting that it may have additional roles in the circulatory systems. Interestingly, although TYMP was initially isolated from platelets, its role in platelet physiology is largely unexplored.

In this study, we examined the hypothesis that TYMP participates in platelet activation and promotes thrombosis. To test this hypothesis, we used a combination of ex vivo studies of human platelets treated with a pharmacological TYMP inhibitor or platelets purified from mice with TYMP deficiency along with in vivo models of arterial thrombosis in genetically modified mice or TYMP inhibitor-treated mice. These approaches show that TYMP contributes to platelet activation and promotes thrombosis in a platelet-dependent manner. We also defined a signaling pathway in which TYMP interacts with Lyn, Fyn, and Yes in platelets to regulate their function.

Methods

All animal uses have been approved by the Institutional Animal Care and Use Committee of The Cleveland Clinic. Methods are available in the Online Data Supplement.

Results

Tymp Deficiency in Mice is Antithrombotic and Platelets Account for the Prothrombotic Role of TYMP

To examine whether TYMP affects thrombosis, 8- to 12-week-old male Tymp +/− and Tymp −/− mice were subjected to the FeCl3-induced carotid artery injury thrombosis model. Male wild-type (WT) mice were used as controls. Figure 1A and Online Movies I to III show representative thrombi formation in the 3 strains over time. Thrombi were confirmed immediately after removing the 7.5% FeCl3 solution saturated filter paper (1 minute in Figure 1A), and no gross differences were observed among the 3 groups. As usual, parts of the initially formed thrombi were washed away by blood flow in all groups (Figure 1A, 2 minutes). Thrombi started to enlarge 3 to 4 minutes after removing the filter paper, and these later thrombi were stable and not washed away. The thrombi in WT mice formed faster than those in Tymp +/− or Tymp −/− mice. Cessation of blood flow was seen in all WT mice (n=9) with an average vessel occlusion time of 11.4 minutes (Figure 1B–1D). Only 3 of the 9 Tymp +/− mice and 4 of the 9 Tymp −/− mice showed flow cessation within the 30 minutes of observation with average occlusion times >20 minutes (Figure 1B–1E). Occlusion times were significantly different between WT and mice with any defect in Tymp, but were not different when comparing Tymp +/− to Tymp −/− mice. These data show TYMP contributes to thrombus formation and suggest that even partial insufficiency of TYMP is enough to provide antithrombotic benefit.

Because TYMP deficiency has been associated with mito-dendrial dysfunction in nucleated human cells, we assessed platelet mitochondrial function by MTT assay. We found no differences in platelet mitochondrial function among the 3 strains (Online Figure IA), which was identical to the original description in this mouse strain. TYMP deletion also did not affect circulating platelet counts (Online Figure IB). TYMP and its ultimate product 2DDRP induce integrin β1 and β3 activation or expression in endothelial cells. We found that TYMP haplo-deficiency or completely deletion did not change the expression of integrin β1 and β3 in platelets; von Willebrand factor levels were also comparable among the 3 strains (Online Figure IC).

Vera et al19 reported that activated platelets secrete 2DDRP, which acts as a cooperative agonist and potentiates platelet activation in response to thrombin. To examine the potential that 2DDRP is low and thus induces antithrombotic phenotype in the TYMP-deficient mice, murine plasma obtained by ultracentrifugation of platelet poor plasma was analyzed by liquid chromatography online tandem mass spectrometry. As shown in Online Figure ID, plasma 2DDRP was similar among the WT, Tymp +/−, and Tymp −/− mice, suggesting that 2DDRP levels are not responsible for the observed phenotype in the Tymp +/− and Tymp −/− mice.

We thus performed bone marrow transplant using Tymp −/− and WT mice to determine the cellular basis of the TYMP-mediated prothrombotic effect. Recipient mice were exposed to 10.5 Gy of external beam irradiation and then infused with 2×1010 donor bone marrow cells by jugular vein injection 4 hours after irradiation. Surviving chimeric mice were subjected to FeCl3-induced carotid artery thrombosis 4 weeks later. The time necessary to form occlusive thrombi was significantly prolonged in WT mice that received Tymp −/− bone marrow compared with WT mice that received WT marrow (Figure 1F); Tymp +/− mice that received WT bone marrow displayed significantly shorter arterial occlusion times when compared with Tymp −/− mice that received Tymp −/− marrow. These data demonstrated that repleting TYMP in circulating cells reverses the antithrombotic effect of systemic TYMP deletion and that TYMP in cells of the vessel wall is not involved in the observed phenotype.

Erythrocytes do not express TYMP so we next distinguished the effect of platelet versus leukocyte TYMP on thrombosis. To do this, platelet transfusion experiments were performed in mice rendered severely thrombocytopenic by irradiation, which decreased platelet counts to 5% of normal after 5 to 6 days. Donor platelets were isolated from Tymp +/−, Tymp −/−, or WT mice, labeled with rhodamine 6G, and then transfused into the thrombocytopenic mouse via jugular vein injection 10 minutes before FeCl3 injury. Each mouse received 105 platelets, which resulted in a final circulating platelet concentration of 6×1011/
L±1.1×10^{11}/L in ≈25 g mice (n=3). Reconstitution of Tymp^{−/−} platelets in Tymp^{+/−} recipient mice resulted in similar occlusion times as nonirradiated Tymp^{+/−} mice. However, thrombocytopenic Tymp^{+/−} mice receiving WT platelets showed an average flow cessation time of 11 minutes, which was significantly shorter than the time to occlusion in thrombocytopenic Tymp^{+/−} mice received Tymp^{−/−} platelets. Similarly, WT recipients transfused with Tymp^{−/−} platelets showed significantly prolonged blood flow cessation time when compared with WT or Tymp^{+/−} animals reconstituted with WT platelets (Figure 1G). These studies demonstrate that platelet TYMP is an important determinant of the antithrombotic phenotype of the Tymp-deficient animals.

**TYMP is Involved in Platelet Activation**

To clarify how TYMP affects thrombosis, we examined agonist-induced platelet aggregation and granule secretion. As shown in Figure 2A, Tymp deletion or haploinsufficiency significantly attenuated ADP-induced platelet aggregation in platelet-rich plasma; the maximum extent of aggregation was reduced ≈33% compared with WT platelets. Tymp deletion nearly abolished platelet aggregation in response to 1 μg/mL collagen (Figure 2B). Tymp haploinsufficiency also significantly blunted platelet aggregation in response to the low-dose collagen, but to a lesser extent than homozygous deletion. We further demonstrated that TYMP deletion significantly decreased thrombin and collagen-related peptide (CRP)-induced aggregation of washed platelet (Figure 2C and 2D). All platelet-activating receptors ultimately trigger platelet α-granule release, resulting in P-selectin translocation to the platelet surface. As shown in Figure 2E, P-selectin expression detected by flow cytometry was significantly attenuated in Tymp-deficient platelets in response to collagen (1 μg/mL), ADP (2.5 μmol/L), thrombin (0.05 U/mL), or CRP (0.5 μg/mL). Tymp deletion also significantly attenuated high-dose agonist-induced platelet activation (Online Figure II). These data suggest that TYMP participates in platelet signaling and activation in response to multiple receptors.

**TYMP Forms a Complex With Src Family Kinases in Platelets**

We previously found that TYMP overexpression induced constitutive phosphorylation of Lyn kinase in vascular smooth muscle cell.2 Lyn is a major Src family kinases (SFK) in human and mouse platelets and plays important and complex roles in platelet activation.22 All SFKs contain an SH3 domain that serves as a site for protein interactions by binding to proline-rich peptide sequences in its binding partners.23 We analyzed the sequence of human TYMP and discovered a consensus SH3-binding sequence APPAP at the N terminus of TYMP.
Figure 2. Tymp deficiency attenuates platelet activation in vitro. A and B, Platelet-rich plasma isolated from Tymp−/−, Tymp+/− or wild-type (WT) mice was used for platelet aggregation assay induced by (A) 2.5 μmol/L ADP and (B) 1 μg/mL collagen using a standard turbidimetric assay. n=6. C and D, Washed murine platelets were used for aggregation assay induced by (C) 0.05 U/mL thrombin or (D) 0.5 μg/mL collagen-related peptide (n=4 or 5). E, Platelets in platelet-rich plasma (20 μL) were mixed with Tyrode buffer (80 μL), and stimulated with indicated agonists for 5 minutes at room temperature. Reactions were stopped by adding 2% formaldehyde, 1 mmol/L EDTA in PBS. P-selectin expression was stained by fluorescein isothiocyanate-conjugated antibody and examined by flow cytometry. *P<0.05 WT vs Tymp+/− and Tymp−/−; #P<0.05, WT vs Tymp−/−, n=3.

Inhibition of TYMP Reduces TYMP/Lyn Association and Glycoprotein VI-Mediated Lyn Activation

Insights gained from structure studies suggest that substrate (or inhibitor) binding induces a conformational change in TYMP, which is essential for its phosphorylase action. Conformation changes are also essential for protein–protein interaction. TYMP metabolites, including thymidine, 2DDR, and 2DDRP did not affect agonist-induced platelet aggregation (Online Figure IV), suggesting that the phosphate and the nucleoside-binding sites of TYMP are not responsible for its effect on platelet function. We thus examined whether another element of the active site of human TYMP, aspartic acid-203, affected its association with SFKs using a potent Asp203-binding TYMP inhibitor, KIN59. As shown in Figure 4A, collagen stimulation significantly increased the amount of TYMP that coprecipitated with Lyn. Inhibition of TYMP with KIN59 significantly decreased the amount of Lyn that coprecipitated with TYMP (Figure 4A and 4B). Immunoblotting of the TYMP precipitates with antibodies specific for phosphorylated Lyn showed that the TYMP-associated Lyn (Figure 4B) was phosphorylated at Y507, an inactive status in resting platelets. On collagen stimulation, TYMP-associated Lyn was rapidly phosphorylated at Y396. However, activity of the TYMP-associated Lyn (detected by phosphorylation at Y396) was only higher at the initial phase (within 1 minute), but was significantly decreased over time (3 minutes). This phenomenon was dramatically blocked by KIN59, suggesting that TYMP may act by trapping and diminishing active Lyn.

Lyn phosphorylates platelet-endothelial cell adhesion molecule 1 (PECAM1, CD31) within its immunoreceptor tyrosine-based inhibitory motif that inhibits platelet activation in response to collagen stimulation. In the presence of the TYMP inhibitor KIN59, we found that inhibition of TYMP activity significantly increased tyrosine-phosphorylated PECAM1 (Figure 4B) was phosphorylated at Y507, an inactive status in resting platelets. On collagen stimulation, TYMP-associated Lyn was rapidly phosphorylated at Y396. However, activity of the TYMP-associated Lyn (detected by phosphorylation at Y396) was only higher at the initial phase (within 1 minute), but was significantly decreased over time (3 minutes). This phenomenon was dramatically blocked by KIN59, suggesting that TYMP may act by trapping and diminishing active Lyn.

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(Figure 3A), which is highly conserved in different primate species. Murine TYMP also contains a core-binding motif PxxP, which is also highly preserved in other orders. These findings suggest that TYMP could directly influence platelet signaling via SFKs or other SH3 domain containing signaling proteins. To test this hypothesis, we first examined whether TYMP interacts with SFKs in platelets. Resting human platelet lysates were immunoprecipitated with specific monoclonal antibodies to TYMP and then the precipitates were assessed for immunoblotting the SFKs, including Src, Lyn, Fyn, and Yes. Normal mouse or rabbit IgG did not pull down any Src, Lyn, Fyn, or Yes, nor TYMP (Online Figure III and not shown). Anti-TYMP precipitates, however, contained Lyn, Fyn, and Yes, but not Src (Figure 3B and 3C). Conversely, anti-Lyn, Fyn, and Yes precipitates also contained TYMP (Figure 3D). These findings were reproduced using platelets from 4 different donors and were also confirmed using mouse platelets (data not shown).
collagen-induced AKT phosphorylation (Figure 4C). To confirm this finding, we pooled platelets harvested from 10 to 12 mice, divided them into 3 groups and then stimulated the platelets with CRP for indicated times. As shown in Figure 4D, in comparison with WT platelets, CRP-induced AKT phosphorylation was dramatically reduced in Tymp\(^{-/-}\) and Lyn\(^{-/-}\) platelets. Tyrosine phosphorylated PECAM1 was significantly increased in Tymp\(^{-/-}\) platelets in response to CRP stimulation, but not in WT and Lyn\(^{-/-}\) platelets. These data suggest that TYMP and Lyn form an association that regulates Lyn function and subsequently affects glycoprotein VI signaling-mediated platelet activation.

**Lyn Deficiency Reverses the Antithrombotic Phenotype of Tymp-Deficient Mice**

Having shown that TYMP interacts with SFKs and regulates SFKs activity in platelets, we hypothesized that deletion of SFK would diminish the antithrombotic phenotype of Tymp deficiency. Yes is not expressed in mouse platelets. Fyn only plays a minor role in mediating glycoprotein VI signaling,\(^{22}\) which plays critical roles in response to vascular injury including the FeCl\(_3\)-induced arterial thrombosis model.\(^{32}\) By platelet transfusion experiments, we found blocking WT platelets with glycoprotein VI antibody significantly prolonged thrombosis time in the 7.5% FeCl\(_3\) injury-induced carotid artery thrombosis model (Figure 5A). Transfusion of Fyn null platelets to the thrombocytopenic WT mice did not affect thrombosis time when compared WT mice received WT platelets (data not shown), supporting that platelet Fyn plays a minor role in these platelets. This data suggest that TYMP and Lyn form an association that regulates Lyn function and subsequently affects glycoprotein VI signaling-mediated platelet activation.

**KIN59 Inhibits Platelet Aggregation In Vitro and Has an Antithrombotic Effect In Vivo Without Affecting Hemostasis**

Having shown that inhibition of TYMP with KIN59 affected activity of platelet signaling molecules, we next examined whether inhibition of TYMP with KIN59 affects platelet activation. KIN59 reversibly and in a dose-dependent fashion inhibited collagen-induced platelet aggregation (Online Figure V). Figure 6A shows cumulative data for different concentrations of KIN59 on different doses of collagen-induced platelet aggregation. These studies demonstrate that inhibition of TYMP activity significantly inhibited platelet aggregation in response to collagen stimulation. Treatment of WT platelets in platelet-rich plasma with KIN59 also dramatically inhibited aggregation in response to 1 \(\mu\)g/mL collagen (Figure 6B), replicating the pattern seen with Tymp\(^{-/-}\) platelets. KIN59 also significantly inhibited aggregation induced by ADP, thrombin, and CRP (not shown).

Given that TYMP plays an important role in platelet activation in response to stimulation by any of examined agonists, we determined whether pharmacological inhibition of TYMP activity affects thrombosis in vivo. KIN59, 30 mg/kg per day, has previously been delivered in vivo to mice and shown to inhibit TYMP-induced angiogenesis\(^{35,36}\) without producing obvious side effects. We treated WT and Tymp\(^{-/-}\) mice with intraperitoneal injection of KIN59 (30 mg/kg/d) for 3 days, and then subjected the mice to FeCl\(_3\)-induced carotid artery injury. Vehicle treatment did not affect carotid artery occlusion times when compared with the untreated WT mice; KIN59 administration, however, significantly prolonged the time to form occlusive thrombus in the WT mice (Figure 6C; Online Figure 3).
Movies IV and V). KIN59 did not further prolong blood cessation time in the Tymp−/− mice (not shown), suggesting that the effect is TYMP dependent.

Hemorrhagic complications are associated with all currently available antiplatelet and anticoagulation therapies. We assessed activated partial thromboplastin time and tail bleeding time to determine whether TYMP deficiency affects hemostasis. Activated partial thromboplastin time was not significantly different among the WT, Tymp+/-, and Tymp−/− mice (Figure 6D). Tymp haploinsufficiency also did not influence bleeding time.

Figure 4. TYMP interacts with Lyn and discriminately regulates phosphorylation of Lyn, platelet-endothelial cell adhesion molecule 1 (PECAM1) (CD31) and AKT in platelets. A, Human platelet lysates were used for immunoprecipitation of Lyn and then immunoblotting was performed for TYMP and Lyn. Blots represent ≥3 independent experiments. P<0.05 vs corresponding times of KIN59 treatments. B, Human platelets were pretreated with vehicle or KIN59 and then stimulated with collagen for indicated times. Platelet lysates were analyzed by immunoprecipitation and immunoblotting assays using indicated antibodies. Bar graph shows Lyn activity from 3 independent experiments. P<0.05 vs corresponding times of KIN59 treatments. C, Human platelets treated as in panel A were analyzed by Western blot using indicated antibodies. Bar graph represents ≥3 independent experiments. D, Mouse platelets pooled from 10 to 12 mice were divided into 3 groups and stimulated with collagen-related peptide as indicated. Platelet lysates were used for immunoprecipitation and immunoblotting assays using indicated antibodies. Bar graph shows the ratio of phosphorylated AKT and PECAM1 to their total proteins.

Figure 5. Lyn deficiency reverses the antithrombotic phenotype of Tymp-deficient mice. A, Platelet glycoprotein VI signaling plays an important role in the FeCl3-induced thrombosis model. The time to blood flow cessation was assessed in Tymp−/−, Lyn−/−, and Tymp−/− mice (n=4 in each group) using the FeCl3 injury induced carotid artery thrombosis model and data were compared with Tymp−/−, Tymp−/− and wild-type (WT) mice.
Bleeding times were prolonged ≈2-fold in Tymp<sup>−/−</sup> mice; however, this level of increase was not associated with any increase in surgical mortality or in observable bleeding at the surgical site. KIN59 treatment for 3 days also did not affect bleeding time (Figure 6E), as well as plasma 2DDRP (Online Figure VI) when compared with the mice received vehicle injection. These data suggest that TYMP may play a role in normal hemostasis, but that haploinsufficiency or significant enzymatic inhibition is not sufficient to cause a hemostatic defect.

**Discussion**

In this study, we demonstrated for the first time that TYMP promotes thrombosis by regulating platelet activation. We found that (1) TYMP deletion or haploinsufficiency in platelets significantly prolonged thrombotic occlusion times in response to FeCl<sub>3</sub>-induced carotid artery injury without affecting hemostasis; (2) TYMP deletion or haploinsufficiency significantly attenuated ADP-, collagen-, CRP-, and thrombin-induced platelet aggregation and α-granule release; (3) in response to platelet agonists, TYMP interacts with Lyn, Fyn, and Yes and modulates activity of Lyn and its downstream signaling molecules, such as PECAM1 and AKT; (4) Lyn haploinsufficiency diminished the phenotype found in the Tymp<sup>+/-</sup> mice; and (5) pharmacological inhibition of TYMP activity reduced agonist-induced human and murine platelet aggregation in vitro and prolonged murine thrombosis times in vivo without affecting hemostasis. This study indicates that targeting TYMP might be a novel antiplatelet or antithrombosis therapy.

TYMP has been found in atherosclerotic plaques, serum of patients with type II diabetes mellitus and cancer. All these diseases have high risk of thrombosis, suggesting that TYMP may play a role in these diseases-associated thrombophilia. This hypothesis is supported by 1 study reported a
decade ago in which increased expression of TYMP in human hepatocellular carcinomas was correlated with high incidence of portal vein thrombosis. This hypothesis is also supported by a recent study, in which perfusion of erythrocyte-encapsulated TYMP to mice resulted in thrombi in the lungs. Interestingly, Aspirin, an antiplatelet drug, inhibits TYMP production in a human monocyte cell line, THP1 cells, suggesting that Aspirin may also inhibit TYMP production in platelet and thus results in the antithrombotic effect. We found that TYMP deficiency did not affect expression of glycoprotein Ib, glycoprotein VI (not shown), von Willebrand Factor or integrin β3 and β, expression in platelets nor was the number of circulating platelets or their mitochondrial function affected by the loss of TYMP. These observations exclude platelet quantity and quality as a source of defective platelet action in the Tymp haploinsufficient or null mice. Activated platelets secrete 2DDRP, which can act as a cooperative agonist and potentiates platelet activation.18

By in vivo thrombosis studies in chimeric mice generated by bone marrow transplant or thrombocytopenic mice reconstituted with WT or Tymp haploinsufficient platelets, we demonstrated that platelet TYMP plays a role in platelet activation and thrombosis, suggesting that TYMP participates in platelet signaling pathway to regulate platelet activation. By analyzing the amino acid sequence of TYMP proteins, we found the N terminus of most mammalian TYMPs contain an SH3 domain-binding proline-rich motif (APPAP in primate) or a core-binding motif (PxxP, in rodents or other species). This finding suggests a molecular mechanism enabling TYMP to participate in signaling by directly binding to SH3 domain containing proteins, such as SFKs. We found that TYMP overexpression induced constitutive Lyn tyrosine phosphorylation in vascular smooth muscle cell. Here, we demonstrate that TYMP forms a complex with Lyn, Fyn, and Yes in human and murine platelets. We found that TYMP associates with Lyn, and that this depends on Asp203-mediated TYMP conformation formation. Furthermore, association of Lyn with TYMP rapidly diminished Lyn phosphorylation at Y396 residue induced by collagen suggesting Lyn activity was inhibited. These data suggest that TYMP acts to sequester active Lyn to decrease Lyn function. Thus, loss of TYMP increases Lyn inhibition of platelet activation.

SFKs share individual and overlapping roles in platelet activation.20 Fyn mainly acts as a stimulation factor; however, Lyn modulates both activation and inhibition of platelet. Lyn and Fyn are constitutively associated with the cytoplasmic domain of glycoprotein VI,46 and Lyn- and Fyn-mediated phosphorylation of the Fc receptor γ chain immunoreceptor tyrosine-based activation motif domain promotes glycoprotein VI-mediated platelet activation. In vitro studies thus showed that Lyn mediates a rapid response to collagen stimulation, especially obvious at low collagen concentrations, and a delayed response of platelets to low doses of collagen-induced aggregation was found in Lyn null platelets.22 We also showed that Lyn functions downstream of CD36 in activating platelets through danger-associated molecular patterns, such as oxidized LDL. Lyn, however, also inhibits collagen-induced platelet activation via phosphorylation of PECAM1 immunoreceptor tyrosine-based inhibitory domain and Lyn-deficient platelets are thus hyper-responsive to collagen stimulation.29 By examining PECAM1 phosphorylation, we found Tymp deletion or inhibition of its activity significantly increased platelet PECAM1 tyrosine phosphorylation in vitro (Figure 4C and 4D). In combination with the fact that TYMP diminishes Lyn phosphorylation at Y396, our data suggest that TYMP attenuates Lyn-mediated PECAM1 immunoreceptor tyrosine-based inhibitory phosphorylation in platelets. TYMP deficiency thus increases Lyn/PECAM1-mediated platelet inhibition and inhibits thrombosis. Our in vivo data suggest this concept as Lyn−/− mice showed a modest acceleration of thrombosis times (Figure 5B, Online Figure VII and Movie VI), and haploinsufficiency of Lyn significantly diminished the antithrombotic phenotype found in Tymp−/− mice. Therefore, our studies indicate that TYMP participates in glycoprotein VI signaling, and regulates Lyn, PECAM1, and phosphoinositol 3-kinase/AKT signaling and platelet function (Figure 7). We also found TYMP influences ADP and thrombin-mediated platelet activation, suggesting a potential role of TYMP in G-protein-coupled receptor signaling. This phenomenon may also because of the effect of TYMP on Lyn activation as Lyn also mediates thrombin-induced and ADP-induced platelet activation.

A human rare autosomal recessive syndrome known as mitochondrial neurogastrointestinal encephalomyopathy has been associated with TYMP loss of function. Patients with this disorder generally die in early adulthood, although at this point no bleeding or thrombotic diathesis has been described. Heterozygous carriers have no discernible phenotype. Indeed, no clinic symptoms of mitochondrial neurogastrointestinal

Figure 7. TYMP functions on platelet glycoprotein VI signaling and promote platelet activation. Red arrows indicate stimulatory and blue arrows indicate inhibitory effects.
encephalomyopathy were present in the Lyn−/− mice.53 Lyn−/− mice generated by a different group also did not show any symptoms of mitochondrial gastrointestineal encephalomyopathy.53 A TYMP inhibitor known as Tipiracil is a component of an experimental anticancer drug TAS-102 and has been used in phase I, II, and III clinical trials. Available data from these studies did not show any bleeding problems or any symptoms similar to the mitochondrial gastrointestineal encephalomyopathy. By examination of activated partial thromboplastin time and tail bleed assays, we found neither Tipiracil deficiency nor inhibition of TYMP with KIN59 affected coagulation hemostasis or bleeding. These data suggest that regulation of TYMP expression or activity is safe and may achieve therapeutic effects.

In summary, our study demonstrated for the first time that TYMP participates in platelet signaling and plays an important role in regulating platelet activation. Genetic deletion of TYMP or pharmacological inhibition of TYMP activity dramatically inhibited platelet activation in vitro and significantly prolonged thrombosis time in vivo. We conclude that pharmacological inhibition of TYMP activity might be a novel approach to develop antplatelet and antithrombotic therapy.

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Disclosures
None.

References
Coimmunoprecipitation studies showed that platelet TYMP interacts with platelet-derived endothelial cell adhesion molecule-1 (PECAM-1), indicating that TYMP overexpression in vascular smooth muscle cell-induced Src family kinase Lyn phosphorylation, which are critical events in clot formation. TYMP regulates collagen-stimulated platelet function by modulating the association of phosphatidylinositol 3-kinase with Grb-2-associated binding protein-1 and linker for activation of T cells. J Thromb Haemost. 2010;8:2530–2541.


Supplemental Material

Methods

All procedures and manipulations of animals have been approved by IACUC of The Cleveland Clinic in accordance with the United States Public Health Service Policy on the Humane Care and Use of Animals, and the NIH Guide for the Care and Use of Laboratory Animals.

Materials: All platelet agonists including adenosine diphosphate (ADP), collagen and thrombin was purchased from Chrono-log (Havertown, PA). Collagen related peptide (CRP) was a gift from Dr. Peter Newman (Blood Research Institute, WI). Antibodies to thymidine phosphorylase (TYMP) (SC-56584 and SC-9523), Lyn (SC-15, SC-7274), Yes (SC-14), Fyn (SC-28791, SC-434), Glycoprotein VI (SC-20149, SC-23551), Actin (SC-1616R) were purchased from Santa Cruz (Dallas, TX). Mouse antibody to TYMP (IC6-203) was a gift from Roche Japan. Antibodies to phosphorylated Lyn (Y396, ab40660) and (Y507, ab33914) were from abcam (Cambridge, MA). FITC-conjugated P-selectin antibody was from BD Biosciences (San Jose, CA). Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) antibody (#3677) was purchased from Cell Signaling (Danvers, MA). Protein G Sepharose 4 Fast Flow was purchased from GE Healthcare Biosciences (Pittsburgh, PA). Horseradish peroxidase (HRP) conjugate of protein G beads was from Life Technologies (Grand Island, NY). Cephalin was from Avanti (Alabaster, Alabama). All other chemical reagents were purchased from Sigma (St. Louis, MO).

Mice: Tymp+/− mouse strain has been back crossed into C57BL6/J (WT) (From the Jackson Laboratory) more than 10 times1; therefore all of the mice used in this study have a homogenous DNA background. Eight to twelve weeks male mice were used in this study. Tymp−/− mouse was bred with Lyn−/− mouse (Gift from Dr. Anthony L. DeFranco), which has been backcrossed at least 15 generations onto C57BL/6 background2, to generate Tymp+/−/Lyn+/− mice. Fyn−/− mice were purchased from Jackson laboratory.

Murine carotid artery thrombosis model: Detailed ferric chloride (FeCl3) induced carotid artery thrombosis model was described before3. Briefly, eight to ten weeks old male mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Injury was induced by topically applying a piece of filter paper (1x2 mm) saturated with 7.5% FeCl3 solution directly on the carotid artery for 1 min. Thrombi formation was observed in real-time using an Intravital Microscope and video images were captured with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2 software (Norpix, Montreal, Canada). The end points were: 1) blood flow has ceased for >30 seconds; or (2) occlusion is not seen after 30 minutes of FeCl3 injury. In this case, the time will be recorded as 30 minutes for statistical analysis.

Mouse platelet isolation: Mice were anesthetized with ketamine/xylazine, 100/10 mg/kg and whole blood were drawn from Tymp−/−, Tymp+/− and WT as well as Lyn−/− and Fyn−/− mice through
inferior vena cava puncture using 0.109 M sodium citrate as anticoagulant\textsuperscript{3,4}. Modified Tyrode’s buffer (in mM: 137 NaCl, 2.7 KCl, 12 NaHCO\textsubscript{3}, 0.4 NaH\textsubscript{2}PO\textsubscript{4}, 5 HEPES, 0.1% glucose and 0.35% BSA, pH 7.2), 0.7 volumes of the whole blood, was added into the whole blood and platelet-rich plasma (PRP) was separated by centrifugation at 100 g for 10 min. Platelets number were counted with hemocytometer. To prepare washed platelets, PRP was centrifuged at 650 g for 6 min in the presence of 0.5 µM PGE\textsubscript{1}. Platelet pellet was resuspended in PBS containing 0.5 µM PGE\textsubscript{1} and recentrifuged (650 g, 6 min). Platelets were then resuspended in PBS, counted and used immediately.

**Platelet MTT assay:** MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to assess mitochondrial function of platelets harvested from the Tymp\textsuperscript{−/−}, Tymp\textsuperscript{+/−} and WT mice. Platelets in PRP (300 µl in 1.5 ml tube) were incubated with MTT (final concentration 1 µg/µl) for 2 hours with gently rotation (30 rotations/min) at 37°C. The platelets were then pelleted by centrifugation (3000 rpm for 5 min) and supernatant was discarded. The platelet pellets were dissolved by adding 350 µl acidified-isopropanol (0.04N HCl in isopropanol) and incubated at room temperature for 15 min with gently rotation. The formazan dye solution was transferred to 96-well plate with triplets (100 µl/well) and read at 570 nm with background subtraction at 630 nm. The O.D. value was adjusted with platelet number.

**Flow cytometry assay:** 2x10\textsuperscript{7} murine platelets in PRP were treated with platelet agonists for 5 min. Equal volume of 2% formaldehyde containing 2 mM EDTA was then added to the reacting solution and platelets were fixed for 30 min at room temperature. The platelets were then washed two times with PBS containing 2 mM EDTA, and resuspended in the same solution. Platelet activation was examined by flow cytometry analysis of P-selectin expression using FITC-conjugated antibody. No stain resting platelets and resting platelets stained with FITC-conjugated P-selection antibody were used as controls.

**Aggregation assay:** Human or murine platelets in PRP were adjusted to a concentration of 2.5 x 10\textsuperscript{8}/ml using platelet-poor-plasma (PPP). Platelet aggregation was performed using a standard turbidimetric assays monitored by an Aggregometer (Chrono-log, Havertown, PA)\textsuperscript{5,7}. Light transmission was monitored over time, and aggregation was quantified as a function of light transmission, with 100% aggregation corresponding to 100% light transmission. In some experiments, platelets were pretreated with KIN59 (in 20% DMSO, 20% cremophore in PBS), a potent TYMP inhibitor, 2 min (or indicated times) before adding calcium/magnesium (final concentration 1 mM) and agonist. Same volume of vehicle (20% DMSO, 20% cremophore in PBS) treated platelets were used as control. To examine GPVI signaling on platelet aggregation, mouse washed platelets were pretreated with GPVI antibody (SC-20149, Santa Cruz) with a final concentration at 2.5 µg/ml or 5 µg/ml for 15 min, and then aggregation was initiated by CRP (0.5 µg/ml).
**Tail bleeding assay:** Eight weeks old Tymp<sup>−/−</sup>, Tymp<sup>+/−</sup> and WT mice was anesthetized (ketamine/xylazine, 100/10 mg/kg) and kept in 37 °C warming chamber. The tails were cut at 1 cm from the tip with a sharp razor blade and immersed into warm saline (37 °C) and cessation time of bleeding was recorded. In another set of study, WT mice received intraperitoneal injection of KIN59 (30 mg/kg) or vehicle for 3 days and then tail bleeding assay was performed to determine whether inhibition of TYMP affects hemostasis.

**Activated partial thromboplastin time (aPTT):** Cephalin was dried under nitrogen gas and resuspended in saline to generate lipid solution with a final concentration of 6.7 mM. Coagulation reagent was prepared in saline containing 0.3% silica and 0.067 mM cephalin. The coagulation reagent as well as 25 mM CaCl<sub>2</sub> solution was pre-warmed to 37°C before use. 100 µl PPP prepared by sequential centrifugation was mixed with 100 µl coagulation reagent in the aggregation tube with stirring (1000 rpm) and incubated at 37°C for 3 min before 100 µl 25 mM CaCl<sub>2</sub> was added. Aggregation was monitored with Chrono-log Aggregometer and time between adding CaCl<sub>2</sub> and start of coagulation was used for statistical analysis.

**Bone marrow transplantation:** 12 weeks old Tymp<sup>−/−</sup> and WT mice were exposed to 10.5 Gy of external beam irradiation from a Cesium 137 source. Four hours later, the recipient mice were anesthetized with ketamine and xylazine (100/10 mg/kg, intraperitoneal injection) for bone marrow injection. To isolate bone marrow, donor mice (12 weeks) were sacrificed by CO<sub>2</sub> asphyxiation. Femurs were dissected free of tissue and flushed with DMEM containing 10% FCS and penicillin/streptomycin (100 U/ml and 100 µg/ml). BM cells were centrifuged and resuspended in saline prior to injection. Each recipient mouse received 10<sup>7</sup> cells via jugular vein injection through a small cervical incision (0.3 cm) under sterile condition. The bleeding was stopped by press the injection site for 2 min and the incision was closed with one simple interrupted suture with an absorbable thread (Ethicon, J571). Four weeks later, the recipient mice with successful bone marrow engrafting were used for the in vivo thrombosis assay using the FeCl<sub>3</sub> induced thrombosis model.

**Platelet transfusion studies:** Tymp<sup>−/−</sup>, Tymp<sup>+/−</sup> or WT mice were exposed to 11 Gy of external beam irradiation to induce thrombocytopenia with platelet counts <5% of normal after 5-6 days<sup>5, 9, 10</sup>. Donor platelets were isolated from WT, Fyn<sup>−/−</sup>, Tymp<sup>+/−</sup> or Tymp<sup>−/−</sup> mice as mentioned above, and stained with Rhodamine 6G (final concentration 50µg/ml) for 15 min at room temperature. Total 10<sup>9</sup> platelets resuspended in 200 µl saline, which brought circulating platelets to a normal range (6 x 10<sup>11</sup>/L ± 1.1 x 10<sup>11</sup>/L in ~25 g mice, n=3)<sup>9</sup>, were injected into the thrombocytopenic mice through the jugular vein 10 minutes prior to FeCl<sub>3</sub> injury.

**Studies using human platelets:** Whole blood was collected from healthy human volunteers in accordance with the Cleveland Clinic IRB. PRP was used for aggregation assays. To establish whether TYMP participates in intracellular signaling pathway, washed human platelets in PBS were pretreated with KIN59 (final concentration 62.5 ~ 250 µM) for 2 min and then stimulated
with collagen (2 µg/ml) for the indicated times. Reactions were stopped by adding EDTA/PGE1 (1 mM/500 nM) solution and platelets were lysed in Pierce IP lysis buffer (in mM: 25 Tris-HCl pH 7.4, 150 NaCl, 1 EDTA, 1% NP-40 and 5% glycerol, Thermo Scientific, Rockford, IL) containing proteinase cocktail (Roche) by three cycles of freezing-thawing action. Unsolvable debris was removed by centrifugation at 12,000 g for 10 min at 4 °C.

**Immunoprecipitation and Immunoblotting assays:** Platelets were harvested, treated and lysed as mentioned above. 500 µg total proteins were used for IP-IB assays using specific antibodies as indicated in the results. For Western blot assay, 30-50 µg total proteins were separated in SDS-PAGE, transferred to PVDF membrane and blotted with indicated antibodies. Some membranes were stripped and re-blotted for actin antibody as loading control.

**Liquid chromatography on-line tandem mass spectrometry (LC/MS) assay of 2DDRP:** Murine whole blood was obtained by inferior vena cava puncture and PPP was obtained by serial centrifugations in the presence of PGE1. The PPP was further ultracentrifuged to remove any cellular residues. The plasma was filtered using Vivaspin 15R Hydrosart columns (2,000Da cut-off) and the filtrates were used for analyzing 2DDRP by LC/MS. Results were presented as pg 2DDRP/gram protein.

**Statistics.** Data were expressed as mean ± SEM. Results were analyzed by 2-tailed Student’s t test or 1-way ANOVA with Bonferroni post-hoc test for multiple comparisons using StatView 5. \( P < 0.05 \) was considered statistically significant.
References


Legends for Online Videos

Online Video I. Representative video of carotid artery thrombus formation in WT mice
Platelets were labeled by direct jugular vein injection of rhodamine 6G. Thrombi formation was initiated by topically applying filter paper (1 x 2 mm) saturated with 7.5% FeCl₃ solution for 1 min and observed in real-time using an Intravital Microscope. Video images were captured with a QImaging Retigo Exi 12-bit mono digital camera and Streampix version 3.17.2 software. White patch indicates thrombi formed. Blood flow became difficulty to be observed over thrombi after 6~7 min. We thus focused on the proximal site (close to heart) to make sure to record accurate time to occlusive thrombi formation.

Online Video II. Representative video of carotid artery thrombus formation in Tymp⁺/⁻ mice. The mice were treated as mentioned in Online Video I.

Online Video III. Representative video of carotid artery thrombus formation in Tymp⁻/⁻ mice. The mice were treated as mentioned in Online Video I.

Online Video IV. Representative video of carotid artery thrombus formation in KIN59 treated WT mice. The mice received TYMP inhibitor KIN59 (in 20% DMSO, 20% cremophore in PBS), 30 mg/Kg/day, intraperitoneal injection for 3 days before they were subjected to the FeCl₃ injury induced thrombosis model on carotid artery.

Online Video V. Representative video of carotid artery thrombus formation in vehicle treated WT mice. The mice received same volume of vehicle, containing 20% DMSO, 20% cremophore in PBS, intraperitoneal injection for 3 days before they were subjected to the FeCl₃ injury induced thrombosis model on carotid artery.

Online Video VI. Representative video of carotid artery thrombus formation in Lyn⁻/⁻ mice. The mice were treated as mentioned in Online Video I.
Supplementary Figure I. *Tymp* deficiency does not affect platelet quantity and quality as well as plasma level of TYMP metabolite 2DDRP. 

**A.** Platelet mitochondrial function was determined by MTT assay, N=5. 

**B.** Platelets in platelet-rich plasma were counted using hemocytometer. N=6. 

**C.** Western blots were performed to analyze expression of the indicated proteins in mouse platelets. 

**D.** Plasma 2DDRP was analyzed with liquid chromatography on-line tandem mass spectrometry (LC/MS). N=3.
Supplementary Figure II. Tymp<sup>−/−</sup> and WT platelets were treated with indicated agonists and P-selectin expression was examined by flow cytometry. N=5, *p<0.05, WT vs. Tymp<sup>−/−</sup>. 
Supplementary Figure III. Human platelet lysate was used for immunoprecipitation (IP) of Lyn or Yes and normal IgG was used as control. The precipitates were then used for immunoblotting (IB) TYMP. “Rb” indicates rabbit, “m” indicates mouse, host animals for developing the antibodies.
Supplementary Figure IV. TYMP metabolites have no effect on agonists induced platelet activation.  

**A.** Human platelet in PRP was used for platelets aggregation assay in the presence of different concentrations of thymidine (n=4).  

**B & C.** 2-deoxy-D-ribose (2DDR) and 2-deoxy-D-ribose-1-phosphate (2DDRP) on collagen induced platelets aggregation (n=3 in C).
Supplementary Figure V. Effect of TYMP inhibitor KIN59 on collagen induced platelet aggregation. A. Collagen (10 μg/ml) induced human platelet aggregation was assessed in the presence of different concentrations of KIN59. B. Platelets were pretreated with 250 μM KIN59 for 5, 15 and 30 min, and then aggregation was initiated with collagen (5 μg/ml). Untreated platelets were used as control.
Supplementary Figure VI. Plasma 2DDRP in the mice treated with KIN59 (30 mg/Kg/day) were examined by LC/MS, n=6.
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Supplementary Figure VII. Representative video images of carotid artery thrombi formation after 7.5% FeCl₃ in WT and Lyn⁻⁻ mice.