

# Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets

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**Tissue factor (TF) is an essential cofactor for the activation of blood coagulation in vivo. We now report that quiescent human platelets express TF pre-mRNA and, in response to activation, splice this intronic-rich message into mature mRNA. Splicing of TF pre-mRNA is associated with increased TF protein expression, procoagulant activity, and accelerated formation of clots. Pre-mRNA splicing is controlled by Cdc2-like kinase (Clk)1, and interruption of Clk1 signaling prevents TF from accumulating in activated platelets. Elevated intravascular TF has been reported in a variety of prothrombotic diseases, but there is debate as to whether anucleate platelets—the key cellular effector of thrombosis—express TF. Our studies demonstrate that human platelets use Clk1-dependent splicing pathways to generate TF protein in response to cellular activation. We propose that platelet-derived TF contributes to the propagation and stabilization of a thrombus.**

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After injury to the vessel wall, platelets adhere to the subendothelial matrix and aggregate with one another to form a primary hemostatic plug (1, 2). Phosphatidyl serine (PS) exposure on the surface of activated platelets provides a thrombogenic surface for the activation of the coagulation protease cascade (3, 4). PS-exposed membranes enhance the catalytic efficiency of membrane-anchored tissue factor (TF) (5), the primary initiator of the coagulation cascade that leads to the conversion of prothrombin to thrombin and fibrinogen to fibrin (6). Whether circulating platelets intrinsically possess TF protein, however, is a debated question (7–12). Studies have demonstrated that monocyte-derived microparticles bind to activated platelets (13–15) and activated platelets themselves release TF-positive microparticles (9, 16, 17).

We report here that platelets from healthy human subjects contain TF pre-mRNA and process it to the mature transcript in response to cellular activation. Activated platelets also

TF protein, procoagulant activity, and accelerate plasma clot formation. The intracellular signaling pathway that controls TF pre-mRNA splicing involves Cdc2-like kinase (Clk)1, an enzyme not previously known to be present or operate in platelets. Inhibition of Clk1 signaling in activated platelets blocks splicing factor 2 (SF2)/alternative splicing factor (ASF) phosphorylation, TF pre-mRNA splicing, and de novo accumulation of bioactive TF protein. Collectively, these data indicate that Clk1-dependent splicing of TF pre-mRNA in platelets is a previously unrecognized pathway to fibrin formation and stabilization of a platelet thrombus.

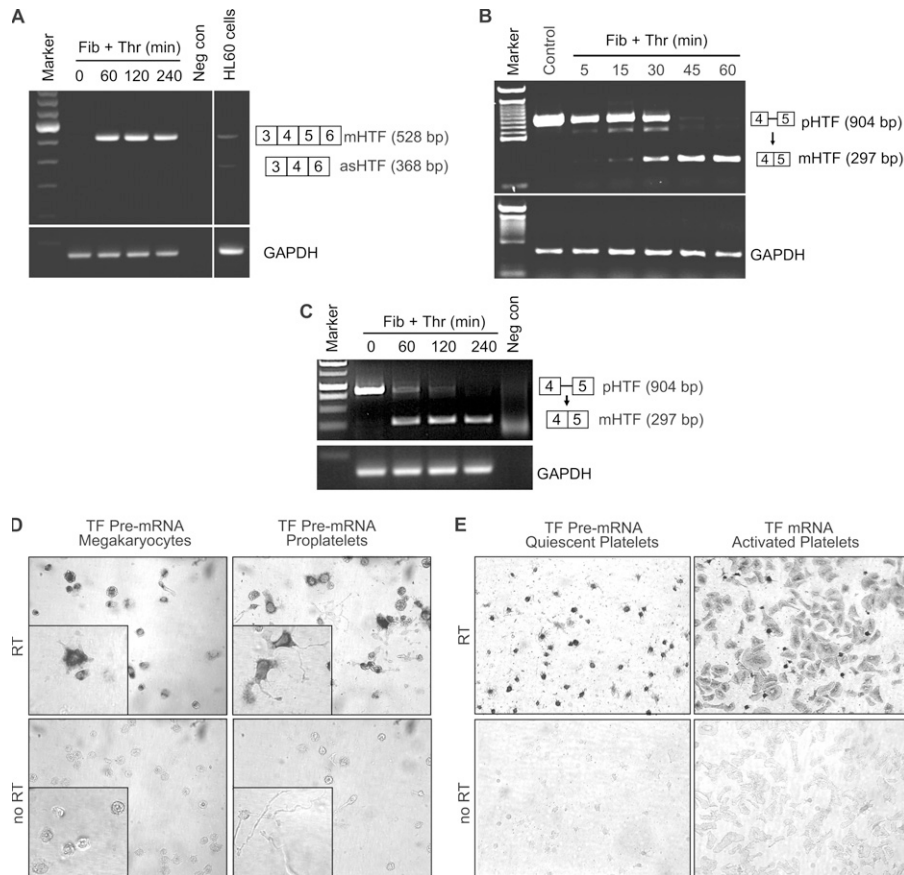
## RESULTS AND DISCUSSION

### Activated platelets splice TF pre-mRNA

To address the question of whether or not platelets express TF, we first determined if platelets contained TF mRNA using highly purified platelet preparations that were isolated from healthy volunteers (reference 18; Supplemental Materials and methods, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>).

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**Figure 1. Platelets contain TF pre-mRNA and splice it into mature message in response to activation.** (A) TF and GAPDH mRNA expression in freshly isolated platelets (0 min), platelets adherent to fibrinogen and coactivated with thrombin (Fib + Thr), or HL60 leukocytes. The negative control lane (Neg con) is a PCR reaction conducted without cDNA template. A is representative of two independent experiments. (B and C) TF and GAPDH mRNA expression in freshly isolated platelets (Control) or platelets that are adherent to fibrinogen in the presence of thrombin (Fib + Thr). The negative control lane (Neg con) in C is a PCR reaction conducted without cDNA template. B and C are

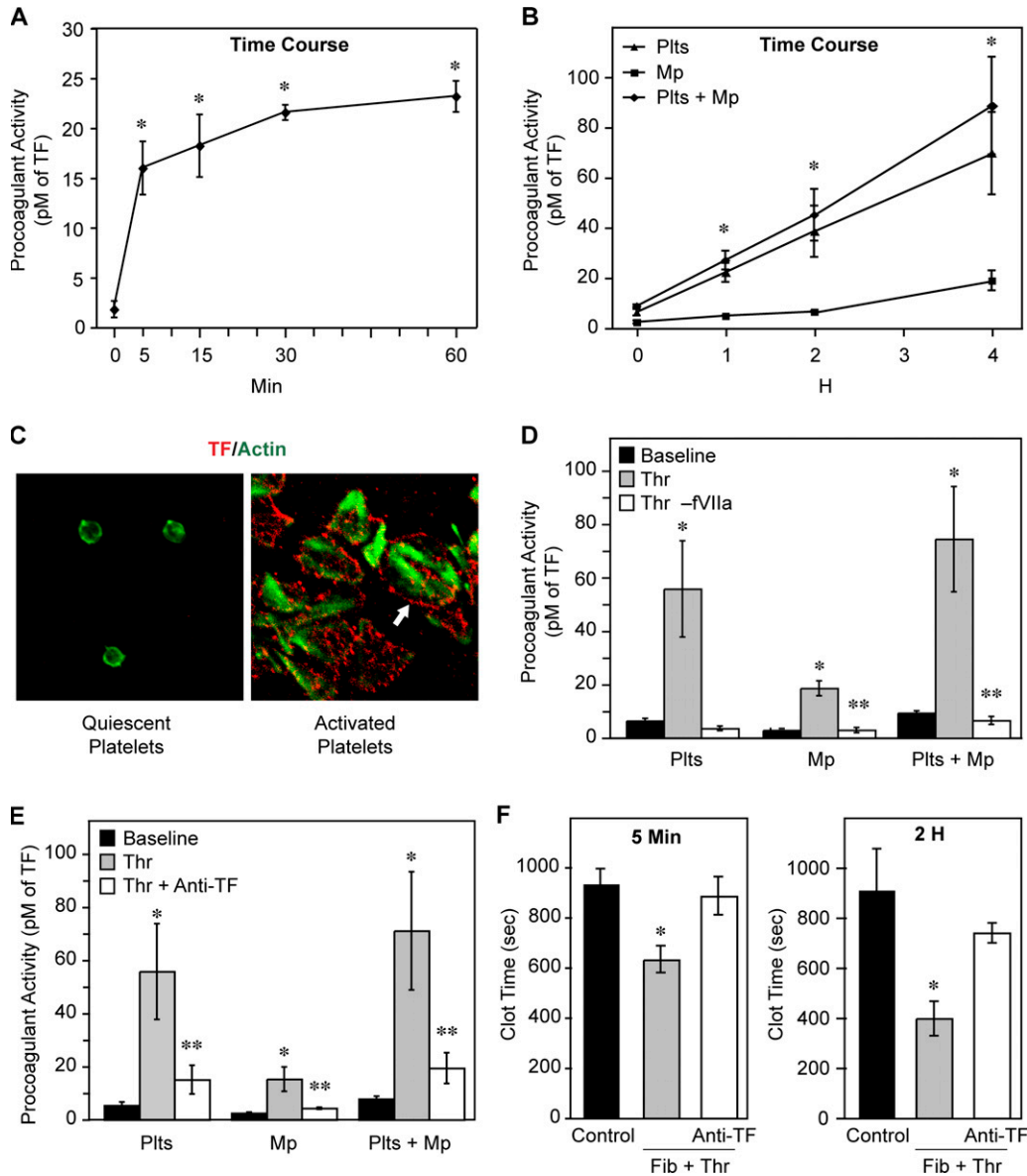
representative of >10 independent experiments. (D) Indirect in situ PCR for intronic TF pre-mRNA in megakaryocytes (left) and megakaryocytes with proplatelet extensions (right). In the bottom panels (no RT), the reverse transcriptase (RT) was omitted from the RT reaction. (E) Direct in situ PCR for TF pre-mRNA was conducted in quiescent platelets (top left), whereas PCR for mature TF mRNA was conducted in platelets adherent to fibrinogen in the presence of thrombin for 1 h (top right). In the bottom panels (no RT), the reverse transcriptase was omitted during the RT reaction. D and E are representative of three independent experiments.

The leukocyte-depleted preparations did not express CD45, PSGL-1, or CD14 (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>, and unpublished data). Unexpectedly, we found that stimulated, but not quiescent, human platelets contain TF mRNA (Fig. 1 A); this was confirmed by amplification of the entire TF message and subsequent cloning of the PCR product (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>, and unpublished data). We did not detect the alternatively spliced variant of TF (19) in stimulated platelets under the conditions of these experiments; the variant was detected, however, in HL60 myeloid leukocytes or resting primary human monocytes (Fig. 1 A and unpublished data).

We surmised that unstimulated platelets contain intronic-rich TF transcripts, a feature that can prevent translation of

the corresponding protein until an appropriate signal induces splicing and assembly of the mature mRNA (18). We designed primer sets that flank intron four and found that freshly isolated platelets predominantly contain TF pre-mRNA (Fig. 1, B and C, see Fig. 4 A, and Fig. S3 A, <http://www.jem.org/cgi/content/full/jem.20061302/DC1>), a finding that was consistent in >40 subjects (unpublished data).

We next determined if platelets activated by fibrinogen and thrombin splice TF pre-mRNA into a mature transcript. Splicing of TF pre-mRNA was detected at 5 min, neared completion by 1 h, and was sustained for at least 4 h after the platelets were activated (Fig. 1, B and C). Other agonists such as ADP, collagen, or thrombin also induce TF pre-mRNA splicing in suspended platelets (Fig. S3). We have recently demonstrated that anucleate platelets possess a functional spliceosome and can splice pre-mRNAs when activated (18),



**Figure 2. Platelet activation increases TF-dependent procoagulant activity.** (A and B) A timecourse (A, 0–60 min; B, 0–4 h) of TF-dependent procoagulant activity in platelets that have adhered to fibrinogen in the presence of thrombin. In B, each line represents procoagulant activity from platelet membranes (Plts), platelet-derived microparticles (Mp), or platelet membranes together with microparticles from the same sample (Plts + Mp). The data are displayed as pM of TF per  $2 \times 10^9$  platelets and represent the mean  $\pm$  SEM of three independent experiments. (C) Immunolocalization of TF in freshly isolated platelets (left) and platelets that have adhered and spread on immobilized fibrinogen in the presence of thrombin for 2 h (right). The green stain in both panels represents actin. The red stain in the right panel shows immunolocalization of TF on the surface of activated platelets (see arrow). This experiment is representative of two independent studies. (D and E) The

bars ( $n = 4$ ) in these panels show TF-dependent procoagulant activity in freshly isolated platelets or platelets activated as in B for 4 h in the presence or absence of factor VIIa (D) or a neutralizing antibody directed against TF (E). A single asterisk (\*) indicates a statistically significant difference ( $P < 0.05$ ) in TF-dependent procoagulant activity between freshly isolated (baseline) and activated platelets; the double asterisk (\*\*) represents a significant difference ( $P < 0.05$ ) between activated platelets under untreated or treated conditions. (F) These panels show clot formation in plasma that is incubated with membranes isolated from quiescent platelets or platelets activated with fibrinogen and thrombin for 5 min (left) or 2 h (right). The white bars represent activated platelets that were incubated with a neutralizing antibody directed against tissue factor (Anti-TF). The data represent the mean  $\pm$  SEM of six independent experiments.

establishing a mechanism for this sequence of events (20). Cloning and sequencing of the unspliced and spliced PCR products confirmed that they were TF (unpublished data).

To unequivocally demonstrate the cell source of pre- and mature TF mRNA species, we screened for the transcripts in individual megakaryocytes, proplatelets, and mature platelets.

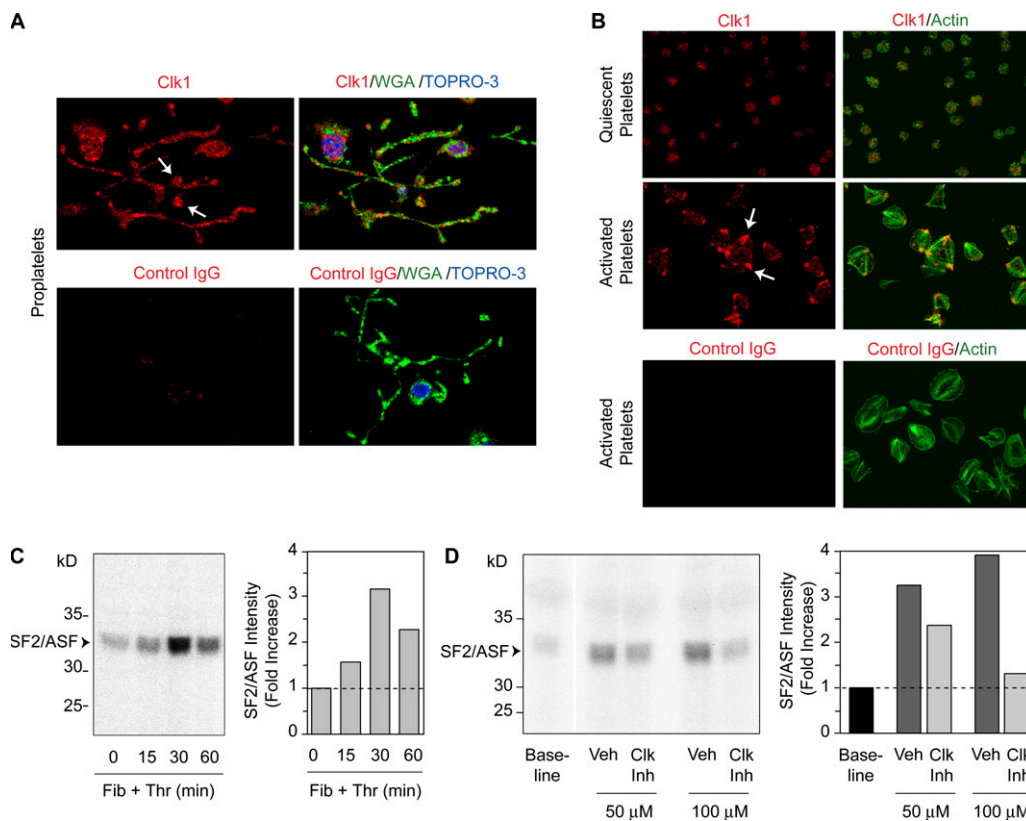
TF pre-mRNA was present in the cytoplasm of hematopoietic stem cell-derived human megakaryocytes and proplatelets (Fig. 1 D). Consistent with detection of intronic-rich message in platelet precursors, we found TF pre-mRNA in freshly isolated platelets from circulating human blood (Fig. 1 E, top left). We also found that activated platelets express TF mRNA (Fig. 1 E, top right), confirming that the mature message is derived from platelets.

**Stimulated platelets generate bioactive TF**

Since stimulated platelets use their splicing machinery to produce mature TF mRNA, we asked if TF-dependent procoagulant activity increased in activated cells. Freshly isolated platelets, at cell numbers ( $2 \times 10^9$  total) that approximate those found in 5–10 ml of whole blood, did not possess significant levels ( $P < 0.05$ ) of procoagulant activity (Fig. 2, A and B). In contrast, procoagulant activity was markedly increased as early as 5 min after platelets adhered to fibrinogen

in the presence of thrombin (Fig. 2 A) and continued to accumulate in platelets and platelet-derived microparticles in a time-dependent fashion (Fig. 2 B). Platelets activated in suspension with ADP, collagen, or thrombin also accumulated TF-dependent procoagulant activity (unpublished data). We consistently observed that activated platelets possessed higher procoagulant activity than quiescent platelets in samples from different donors, but the magnitude was variable among subjects, ranging from a 2.8- to a 15.3-fold increase over baseline (mean increase over baseline  $7.7 \pm 2.0$ ). In contrast to activated platelets, monocytes ( $5 \times 10^6$ ) stimulated with fibrinogen and thrombin did not generate appreciable procoagulant activity, although they did respond to lipopolysaccharide (unpublished data).

Next, we analyzed the protein by immunocytochemistry and observed TF on the surfaces of activated platelets that had adhered and spread on immobilized fibrinogen in the presence of thrombin but not on quiescent platelets (Fig. 2 C).



**Figure 3. Platelet Clk1 induces SF2/ASF phosphorylation.** (A) Megakaryocytes with proplatelet extensions were stained with anti-Clk1 (top) or control IgG (bottom). The right panels are overlays where anti-Clk1 or IgG are in red, wheat germ agglutinin is in green, and topo-3, which stains nuclei, is in blue. (B) Platelets were left quiescent (top) or activated by adherence to immobilized fibrinogen in the presence of thrombin for 1 h (middle and bottom). The cells were stained with anti-Clk1 (top and middle) or control IgG (bottom). The right panels are overlays in which anti-Clk1 or IgG is in red, polymerized actin is in green, and colocalization of the two markers is identified by yellow staining. A and B are represen-

tative images of multiple cells taken from three and four independent experiments, respectively. (C and D) Platelets were activated as in B, lysed, and immune complex kinase assays for Clk1 activity were performed with recombinant SF2/ASF as the substrate. C shows a time course of Clk1-dependent SF2/ASF phosphorylation in activated platelet lysates. D shows Clk1-dependent SF2/ASF phosphorylation in freshly isolated platelet lysates or platelets that were pretreated with vehicle or Tg003 (Clk Inh) (lanes 2–5). The bars for C and D represent fold increases in SF2/ASF phosphorylation over baseline as estimated by densitometry. The gels in C and D are representative of three independent experiments.

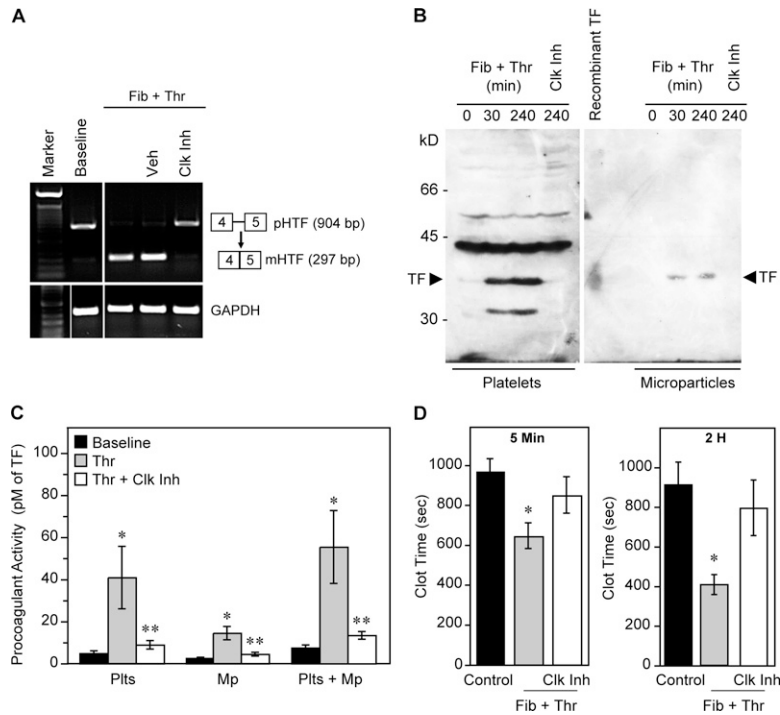
Staining was detected on the surface of all the platelets, consistent with the detection of TF mRNA in every cell (Fig. 1 E, top right).

Deletion of factor VIIa from the activity assay or incubation of platelets with a neutralizing anti-TF antibody significantly ( $P < 0.05$ ) reduced procoagulant activity in stimulated platelets (Fig. 2, D and E). This activity assay, however, evaluates factor Xa generation in the presence of supraphysiologic levels of exogenous factor VIIa (7). Therefore, we determined if platelet-derived TF decreased plasma clotting times. We found that human plasma incubated with platelets for 5 min or 2 h formed clots at an accelerated rate compared with freshly isolated platelets, which by themselves had no appreciable effect on plasma clot formation (Fig. 2 F). Clotting was significantly delayed in the presence of an inhibitory anti-TF antibody (Fig. 2 F), indicating that TF generated by activated platelets is capable of accelerating

in vitro clot formation in humans. Collectively, the studies depicted in Fig. 2 demonstrate that bioactive TF protein accumulates in platelets adherent to fibrinogen in the presence of thrombin. The role of platelet-derived TF in the propagation and stabilization of platelet clots in vitro and in vivo will require further studies using both human and murine thrombosis models.

#### Clk1 interfaces with the splicing machinery in platelets

The intracellular signaling pathways that control TF pre-mRNA splicing and activity in platelets are not known (18). In nucleated cells, serine-arginine (SR)-rich proteins regulate splicing, and we recently found that human platelets contain the SR family member SF2/ASF (18). Thus, we considered that platelets possess critical upstream kinases that regulate SF2/ASF activity and focused on Clk family members because one of them, Clk1, contains an N-terminal



**Figure 4. Interruption of Clk1 blocks signal-dependent TF pre-mRNA splicing and bioactive protein accumulation in platelets.**

(A) Human TF pre-mRNA (pHTF; 904 bp) and mature mRNA (mHTF; 297 bp) was determined in freshly isolated platelets and in platelets adherent to immobilized fibrinogen and coactivated with thrombin for 2 h. The activated platelets were either left untreated (lane 3) or pretreated with vehicle (Veh) or Tg003 (Clk Inh). (B) Western blot analysis showing TF protein expression in platelets and platelet-derived microparticles. The platelets were left quiescent (baseline) or adhered to immobilized fibrinogen and coactivated with thrombin for 30 min or 4 h in the presence or absence of Tg003 (Clk Inh). Recombinant TF was used as a positive control. The panels in A and B are representative of at least three independent experiments. (C) This panel depicts TF-dependent procoagulant activity in freshly isolated platelets or platelets activated, as in Fig. 2 B, in the presence or absence of the Clk inhibitor. The data are displayed as pM of TF per  $2 \times 10^9$

platelets and represent the mean  $\pm$  SEM of six independent experiments. A single asterisk (\*) indicates a statistically significant difference ( $P < 0.05$ ) in TF-dependent procoagulant activity between freshly isolated (baseline) and activated platelets; the double asterisk (\*\*) represents a significant difference ( $P < 0.05$ ) between activated platelets under untreated or treated conditions. (D) These panels show plasma clot formation in the presence of membranes isolated from freshly isolated platelets (Control) or platelets activated for 5 min (left) or 2 h (right) with fibrinogen and thrombin. The activated platelets were pretreated with either the Clk inhibitor (Clk Inh) or DMSO. The data represent the mean  $\pm$  SEM of five independent experiments. The single asterisk (\*) indicates a statistically significant difference ( $P < 0.05$ ) in the rate of clot formation in plasma samples exposed to fibrinogen and thrombin-treated platelets compared with quiescent platelets (Control) and activated platelets treated with the Clk inhibitor.

region enriched in arginine-serine dipeptides (RS) that interacts with SF2/ASF (21). We found Clk1 protein in the cytoplasm of mature megakaryocytes (unpublished data), in proplatelets that extend from the megakaryocytes (Fig. 3 A, arrows), and in quiescent circulating platelets from human blood (Fig. 3 B, arrows). In activated platelets, Clk1 was distributed to the tips of F-actin stress cables (Fig. 3 B). Intracellular redistribution of Clk1 resembles the accumulation of vinculin in focal adhesion complexes of platelets that are spread and activated on immobilized fibrinogen (unpublished data and reference 22). Preliminary screens for other family members (Clk2, 3, and 4) were negative (unpublished data), suggesting that Clk1 is the primary Clk in mature, circulating platelets.

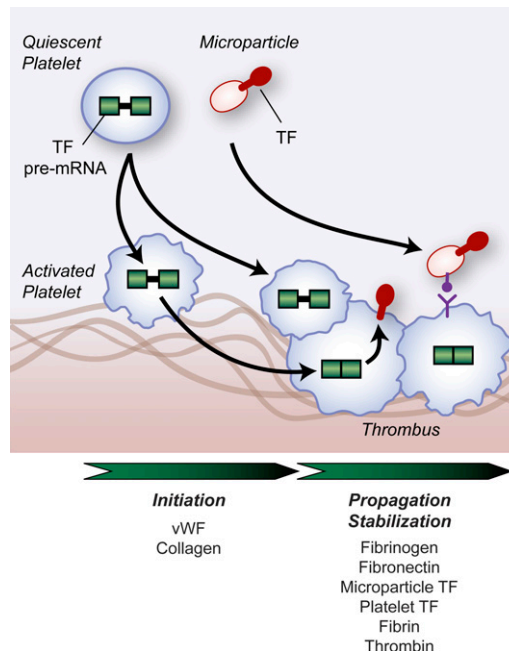
In nucleated cells, Clk1 directly phosphorylates SF2/ASF and alters the intracellular localization patterns of SR proteins (21). Therefore, we captured endogenous platelet Clk1 by immunoprecipitation and determined if it regulates SF2/ASF phosphorylation. Clk1 from activated platelets markedly increased SF2/ASF phosphorylation (Fig. 3 C). Increased SF2/ASF phosphorylation was not seen when control IgG was used as the immunoprecipitating reagent (Fig. S4 A, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>).

A benzothiazole compound, Tg003, suppresses Clk1-catalyzed phosphorylation and thereby inhibits SF2/ASF-dependent splicing of *in vitro*-transcribed pre-mRNAs in immortalized cell lines (23). In stimulated platelets Tg003, but not its vehicle, suppressed Clk1-dependent SF2/ASF phosphorylation (Fig. 3 D), consistent with previous characterization of the inhibitor in other cells (23).

#### Clk1 controls the expression of TF in activated platelets

We next asked if interruption of signaling from Clk1 to SF2/ASF modulates activation-dependent splicing and found that Tg003 prevented processing of TF pre-mRNA in activated platelets (Fig. 4 A). This Clk1-dependent splicing inhibitor also blocked the expression of TF protein in activated platelets and platelet-derived microparticles (Fig. 4 B). Consistent with its effect on protein, Tg003 significantly ( $P < 0.05$ ) reduced TF-dependent procoagulant activity in stimulated platelets (Fig. 4 C) and delayed the onset of plasma clot formation (Fig. 4 D). Puromycin, an inhibitor of mRNA translation, also significantly ( $P < 0.05$ ) reduced TF-dependent procoagulant activity demonstrating the increases were caused by *de novo* protein synthesis (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>).

Although Tg003 blocked SF2/ASF phosphorylation (Fig. 3 C) and the expression of TF protein in activated platelets (Fig. 4), it had no effect on other platelet functional responses that included cellular adherence and spreading, actin polymerization, organization of  $\beta$ -tubulin, or the redistribution of Clk1 to focal adhesion complexes (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>). These data suggest that the Clk1 signaling pathway primarily interfaces with the splicing machinery in platelets.



**Figure 5.** Proposed model by which platelet-derived tissue factor contributes to propagation and stabilization of a thrombus.

#### Conclusions

Pre-mRNA splicing and regulated translation of processed mRNAs are novel functions that allow activated platelets to alter their transcriptome and proteome in response to stimulation (18, 20, 24). IL-1 $\beta$  was the first platelet product discovered to be synthesized in this fashion (18). In this report, we demonstrate that platelets also use their splicing machinery to control the expression of TF and identify a new intermediate, Clk1, in the signaling pathway leading to TF synthesis. Our results indicate that quiescent platelets contain TF pre-mRNA but do not express significant levels ( $P < 0.05$ ) of TF protein or activity under basal conditions. In contrast, activated platelets express both TF mRNA and bioactive TF protein. Pre-mRNA splicing and translation of TF message into protein is observed as early as 5 min after activation and is sustained for at least 4 h. The time scale of this response suggests that platelet-derived TF sustains the growth of the thrombus and increases its stability by enhancing fibrin deposition (Fig. 5). Formation of a stable thrombus is essential for hemostasis and promotes wound healing at the site of vascular injury. Recent studies have found that mice with deficiencies in TF or fibrinogen form unstable thrombi (25, 26). Tissue factor also modulates inflammation and angiogenesis (27, 28), indicating that it affects prolonged functional responses. At present the relative contributions of platelet-derived TF and TF-positive microparticles to thrombus formation under different pathologic conditions are not known. Although on a per-cell basis lipopolysaccharide-stimulated monocytes generate greater amounts of TF than platelets (unpublished observations), the number of circulating platelets far exceed (i.e.,  $\sim 500$ – $1,000$ -fold greater) the number of

monocytes per volume of blood. In the case of platelets, our studies are the first to demonstrate that Clk1 modulates TF gene expression and suggest that splicing of TF pre-mRNA may be a potential therapeutic target in syndromes of disordered coagulation.

## MATERIALS AND METHODS

**Cells.** CD34<sup>+</sup> stem cells were isolated from human umbilical cord blood and were differentiated into megakaryocytes that produce proplatelets using methods that we have previously described (18). Leukocyte-depleted human platelets were isolated from healthy volunteers using previously described methods (18, 29). The human studies were approved by the University of Utah Internal Review Board (IRB approval numbers 392 and 11919).

**mRNA detection systems.** For most of the studies, primers that targeted sequences in exon four (5'-CTCGGACAGCCAACAATTCAG-3') and five (5'-CGGGCTGTCTGTACTCTTCC-3'), and thus spanned intron four, were used to determine endogenous splicing of TF pre-mRNA in platelets. Indirect in situ hybridization or direct in situ PCR was used to detect TF pre-mRNA in megakaryocytes and platelets as previously described (18).

**Protein detection systems.** Detailed strategies for protein detection by flow cytometry, Western blot analysis, and immunocytochemistry have been previously published (18, 29).

**Measurement of TF-dependent procoagulant activity.** TF-dependent procoagulant activity was calculated with an Actichrome TF assay (American Diagnostica) as previously described (13).

**Plasma clotting.** Platelets were left quiescent or activated in the presence or absence of Tg003. Platelet membranes were isolated and added to human plasma (37°C), and clotting was initiated with CaCl<sub>2</sub> as previously described (7).

**Immune complex kinase assay for Clk1 activity.** Clk1 activity in platelets was determined using an immune complex kinase assay. An antibody against Clk1 was used for immunoprecipitation of the protein. Nonimmune rabbit IgG was used as a control, and in select experiments recombinant SF2/ASF was removed from the assays to screen for nonspecific incorporation of radiolabeled phosphate (Fig. S4, A and B). Kinase assays were performed by addition of recombinant SF2/ASF (Protein One) in the presence of  $\gamma$ -[<sup>32</sup>P]ATP (MP Biomedicals). At the end of this incubation period, the agarose beads and immune complexes were removed by centrifugation, and the unbound sample, which contained SF2/ASF, was resolved by SDS-PAGE.

**Statistical analyses.** ANOVA was conducted to identify differences that existed among multiple experimental groups. If significant differences were found, a Student-Newman-Keuls post-hoc procedure was used to determine the location of the difference. For all of the analyses,  $P < 0.05$  was considered statistically significant.

**Online supplemental material.** Supplemental Materials and methods details methods for cellular activation, in situ hybridization, and protein detection. Fig. S1 provides data from flow cytometric analysis for CD45 or control IgG in leukocyte-depleted platelets or monocytes. Fig. S2 shows that activated platelets express full-length, mature (mHTF) human TF mRNA. Fig. S3 shows TF pre-mRNA splicing in human platelets due to activation with ADP, collagen, and thrombin in suspension. Fig. S4 provides additional data regarding the specific Clk1-mediated SF2/ASF phosphorylation. Fig. S5 shows that the inhibition of translation prevents activated platelets from generating bioactive tissue factor. Fig. S6 shows that inhibition of Clk1 does not adversely affect platelet functional responses. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061302/DC1>.

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