

## Association of Factor V Secretion with Protein Kinase B Signaling in Platelets from Horses with Atypical Equine Thrombasthenia

J.W. Norris, M. Pombo, E. Shirley, G. Blevins, and F. Tablin

**Background:** Two congenital bleeding diatheses have been identified in Thoroughbred horses: Glanzmann thrombasthenia (GT) and a second, novel diathesis associated with abnormal platelet function in response to collagen and thrombin stimulation.

**Hypothesis/Objectives:** Platelet dysfunction in horses with this second thrombasthenia results from a secretory defect.

**Animals:** Two affected and 6 clinically normal horses.

**Methods:** Ex vivo study. Washed platelets were examined for (1) expression of the  $\alpha$ IIB- $\beta$ 3 integrin; (2) fibrinogen binding capacity in response to ADP and thrombin; (3) secretion of dense and  $\alpha$ -granules; (4) activation of the mammalian target of rapamycin (mTOR)-protein kinase B (AKT) signaling pathway; and (5) cellular distribution of phosphatidylinositol-4-phosphate-3-kinase, class 2B (PIK3C2B) and SH2 containing inositol-5'-phosphatase 1 (SHIP1).

**Results:** Platelets from affected horses expressed normal amounts of  $\alpha$ IIB- $\beta$ 3 integrin and bound fibrinogen normally in response to ADP, but bound 80% less fibrinogen in response to thrombin.  $\alpha$ -granules only released 50% as much Factor V as control platelets, but dense granules released their contents normally. Protein kinase B (AKT) phosphorylation was reduced after thrombin activation, but mTOR Complex 2 (mTORC2) and phosphoinositide-dependent kinase 1 (PDK1) signaling were normal. SH2-containing inositol-5'-phosphatase 1 (SHIP1) did not localize to the cytoskeleton of affected platelets and was decreased overall consistent with reduced AKT phosphorylation.

**Conclusions and clinical significance:** Defects in fibrinogen binding, granule secretion, and signal transduction are unique to this thrombasthenia, which we designate as atypical equine thrombasthenia.

**Key words:** Factor V; AKT; Bleeding Diathesis; Horse.

Two bleeding diatheses resulting from inherited platelet dysfunctions have been identified in horses. In both diseases, platelets lack the capability to bind fibrinogen in response to some or all physiological agonists of platelet activation. However, the underlying molecular defects leading to aberrant fibrinogen binding differ between these disorders.

Glanzmann thrombasthenia (GT) was the first bleeding diathesis identified in horses,<sup>1</sup> and mutations have been identified in exons 2 and 11 of  $\alpha$ IIB gene of the fibrinogen receptor.<sup>2–5</sup> Both mutations are inherited in an autosomal dominant mode and either mutation prevents platelets from properly expressing the  $\alpha$ IIB- $\beta$ 3 integrin. Like the homologous human disease, platelets from horses with GT fail to bind fibrinogen; aggregate in response to physiological agonists, including ADP;

*From the 5A60 Johns Hopkins Asthma and Allergy Center, Johns Hopkins Medical Institute – Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD (Norris); Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA (Pombo, Tablin); Hunters Glen Veterinary Hospital, Inc., Veterinary Acupuncture Services of Tulsa LLC, Tulsa, OK (Shirley); and the 19463 James Monroe HWY, Leesburg, VA (Blevins).*

*All work was performed at the University of California-Davis.*

*Corresponding author: F. Tablin, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, One Shields Ave., Davis, CA 95616; e-mail: ftablin@ucdavis.edu.*

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### Abbreviations:

GT	glanzmann thrombasthenia
AET	atypical equine thrombasthenia
AKT	protein kinase B
GSK	glycogen synthase kinase
mTOR	mammalian target of rapamycin
mTORC2	mTOR complex 2
PDK1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PI(3,4)P <sub>2</sub>	phosphatidylinositol 3',4'-bisphosphate
PI(3,4,5)P <sub>3</sub>	phosphatidylinositol 3',4',5'-trisphosphate
PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase, class 2 $\beta$
PKC	protein kinase C
PLC $\beta$	phospholipase C $\beta$
PRP	platelet-rich plasma
RGDS	arginine-glycine-aspartate-serine
S473	serine 473
SHIP1	SH2-containing inositol-5'-phosphatase 1
T308	threonine 308
T <sub>R</sub>	room temperature
TX-100	Triton X-100

or retract blood clots.<sup>1,6</sup> Although it has not been measured in equine patients, human patients with GT generate thrombin normally on their surfaces after activation.<sup>7</sup> The identification of a Thoroughbred-cross with homozygous mutations in exon 2 suggests that GT has been present in Thoroughbred lineages.<sup>1</sup>

The second bleeding diathesis in Thoroughbreds was characterized in a filly, which subsequently transmitted the trait to 1 of 2 offspring.<sup>8,9</sup> Aggregation of platelet rich plasma (PRP) from affected horses is delayed in response to collagen, and washed platelets bind markedly less fibrinogen in response to thrombin compared to normal platelets. Three additional Thoroughbred horses that share this platelet phenotype were subse-

quently identified at a breeding farm in Northern California.<sup>10</sup> Unlike horses with GT, platelets from horses with the second bleeding diathesis aggregate in response to ADP and retract blood clots, but have markedly reduced thrombin production despite normal externalization of phosphatidylserine.<sup>8,9</sup>

We hypothesized that platelets from horses with this second thrombasthenia have defective secretion of platelet Factor V, which is necessary for assembly of the prothrombinase complex on the surface of activated platelets and normal thrombin production. To evaluate the contribution of integrins to this platelet defect we determined the amounts of both integrin subunits and their fibrinogen binding capacity under conditions of minimal granule secretion in comparison to control platelets, and observed no differences. In contrast, Factor V secretion by platelets from affected horses was reduced in comparison to normal control platelets, consistent with decreased thrombin production. After thrombin stimulation, phosphorylation of protein kinase B (AKT) at serine 473 has been shown to be necessary for normal fibrinogen binding and secretion by murine platelets.<sup>11</sup> Similarly, we identified a novel defect in signal transduction associated with the mammalian target of rapamycin (mTOR)-AKT pathway in platelets from horses with this second thrombasthenia. As a naturally occurring bleeding diathesis with these characteristics has not been identified, we formally designate this disease in horses as atypical equine thrombasthenia (AET).

## Materials and Methods

### Animals

Two affected and 6 clinically normal horses were housed at the Center for Equine Health, University of California-Davis. Blood was collected into ACD-A Vacutainer tubes by jugular venipuncture under an institutionally approved protocol.

### Platelet Preparation

Within 60 minutes of collection, blood was centrifuged in  $17 \times 120$  mm conical, polypropylene tubes ( $200 \times g$ , 15 minutes, room temperature [ $T_R$ , 25°C]) as previously described.<sup>9</sup> Platelet-rich plasma was transferred to  $17 \times 100$ -mm round-bottom, polypropylene tube. Either 0.1 U/mL apyrase<sup>a</sup> and 1 mM EDTA<sup>11</sup> or 10 µg/mL PGE<sub>1</sub><sup>b</sup> were added to PRP for either Western blot or flow cytometry studies, respectively. PRP was then centrifuged ( $400 \times g$ , 15 minutes,  $T_R$ ). The pellet was suspended in 10 ml Tyrode's-HEPES (12 mM NaHCO<sub>3</sub>, 138 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 10 µg/mL PGE<sub>1</sub>, pH 7.2); inhibitors were added, as described above; and the sample was centrifuged a second time with the same parameters. After suspension in Tyrode's-HEPES at concentrations listed below for each experiment, CaCl<sub>2</sub> was added to platelets in 5 equal aliquots at 5 minute intervals from a 10 mM stock in Tyrode's-HEPES such that the final concentration was 2 mM CaCl<sub>2</sub> and  $5 \times 10^7$  cells/mL. Platelets prepared in this manner are referred to as "washed platelets" throughout the remainder of the text. All platelet counts were determined using an automated blood counter.<sup>c</sup>

### Fibrinogen Binding

Washed platelets ( $5 \times 10^7$ /mL) preincubated with Alexa 488-labeled human fibrinogen<sup>d</sup> were activated with either ADP (50 µM)<sup>e</sup> or

$\alpha$ -thrombin (0.1NIH-U/mL)<sup>f</sup> and fluorescence was measured by flow cytometry.<sup>9</sup> All data were collected with an FC500 flow cytometer.<sup>g</sup> Forward and side scatter voltages were set to detect machine noise, which was removed during subsequent analyses. The FL1 detector was set to 500 V to prevent saturation of the detector. Specific fibrinogen binding, calculated as the difference between fluorescence of resting and ADP-activated platelets, was used to determine platelet fibrinogen binding parameters.<sup>12</sup>

### Western Blotting

Except as described below, washed platelets at  $2 \times 10^8$ /mL were activated with  $\alpha$ -thrombin (0.1NIH-U/mL) at 37°C. Protease inhibitors (4 mM AEBSF, 1 µM leupeptin, 1 µM pepstatin, 0.3 µM aprotinin, and 100 µg/mL soybean trypsin inhibitor, final concentrations)<sup>h</sup> and  $2 \times$  Laemmli buffer (20% glycerol, 4% SDS, 20 mM EDTA, 200 mM dithiothreitol,<sup>i</sup> and 50 mM tris[hydroxymethyl]aminomethane, pH 6.8) were added at indicated times. Lysates were denatured (95°C, 5 minutes); resolved by 7% SDS-PAGE ( $3 \times 10^6$  platelet equivalents/lane); transferred to nitrocellulose<sup>j</sup>; and incubated with indicated primary and appropriate secondary antibodies<sup>k</sup> before chemiluminescent imaging<sup>l</sup> and quantification.<sup>m</sup>

### Secreted Factor V

Supernatants containing releasates from washed platelets ( $1 \times 10^9$ /mL) activated with  $\alpha$ -thrombin (1.0NIH-U/mL, 10 minutes 37°C) were separated by centrifugation ( $3,000 \times g$ , 5 minutes,  $T_R$ ). Releasates were evaluated by Western blot, as described above, using  $1.5 \times 10^7$  platelet equivalents of releasate per lane.

### Mepacrine Retention

Washed platelets ( $1 \times 10^7$ /mL) were incubated with mepacrine (10 µM final, 1 hour, 37°C), before activation with  $\alpha$ -thrombin (10 minutes, 37°C). Reactions were diluted 1 : 100 in Tyrode's-HEPES containing 2 mM CaCl<sub>2</sub> and fluorescence evaluated by flow cytometry with the FL1 detector voltage set as described previously for fibrinogen binding. Around 10,000 platelets were evaluated per sample.

### Preparation of Triton X-100 (TX-100) Soluble and Insoluble Fractions

About  $10 \times$  Lysis Buffer (10% TX-100, 40 mM AEBSF, 10 µM leupeptin, 10 µM pepstatin, 3 µM aprotinin, 200 mM EDTA, and 1 mg/mL soybean trypsin inhibitor) was added to washed platelets activated with  $\alpha$ -thrombin, as described for Western Blotting. Samples were incubated on ice for 30 minutes and then centrifuged ( $21,000 \times g$ , 10 minutes, 4°C). Supernatants were collected and pellets washed once with  $1 \times$  Lysis Buffer diluted with Tyrode's-HEPES. After centrifugation, pellets were resuspended with  $1 \times$  Lysis Buffer to equal volumes as the supernatants. Both were prepared for Western blot analysis as described above. Where indicated, platelets were incubated with 0.4 mM arginine-glycine-aspartate-serine (RGDS, 1 minutes,  $T_R$ ) before activation.

### Statistical Analysis

Means and standard deviations represent 3 independent trials. Statistical significance of differences was determined by 2-tailed *t*-test, with  $P < .05$  considered significant.<sup>n</sup>

## Results

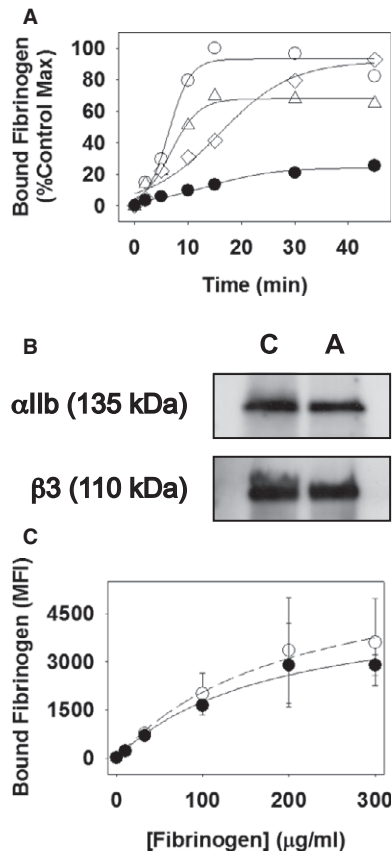
Fibrinogen binding to AET platelets from the index case reached saturation within 30 minutes, similar to control horses. However, AET platelets bind only approximately 20% as much fibrinogen (Fig 1A). The  $\alpha$ IIb and  $\beta$ 3 integrin subunits of the fibrinogen receptor were present at comparable amounts in platelets from control and affected horses (Fig 1B). Specific fibrinogen binding to platelets from both normal horses and the index case was saturable (Fig 1C), and binding parameters determined were identical between groups (Table 1).

Factor V, which is released from platelet  $\alpha$ -granules, was found to be present at equivalent levels in resting platelets from control and affected horses (Fig 2A). Supernatants from purified, resting platelets did not

contain detectable amounts of Factor V. In response to  $\alpha$ -thrombin, significantly less Factor V was released from AET platelet  $\alpha$ -granules as compared to controls ( $47.2 \pm 14.4\%$ ,  $P < .001$ ). However, the fluorescent dye mepacrine, which is a marker of dense granule secretion,<sup>13</sup> was released identically from all horses' platelets at all thrombin concentrations tested (Fig 2B).

There were identical levels of AKT1 and AKT3 in platelets from normal and affected horses throughout a 10 minute time course after  $\alpha$ -thrombin activation (Fig 3A). Protein kinase B isoform 2 (AKT2) was not detected in equine platelets. Antibodies to AKT3 bound a single protein in resting platelets and for the first 30 seconds after activation, but bound 2 proteins of close, but distinct, molecular weights for the remainder of the time course.

Protein kinase B is activated by phosphorylation at Serine 473 (S473), which reached a maximum in control platelets 8 minutes after  $\alpha$ -thrombin activation and

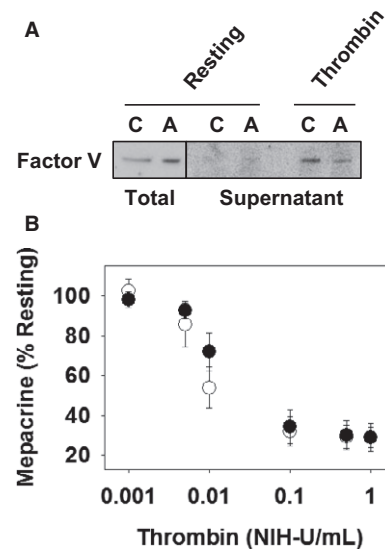


**Fig 1.** Fibrinogen binding by atypical equine thrombasthenia (AET) platelets is reduced after stimulation with  $\alpha$ -thrombin despite normal fibrinogen binding capacity. (A) Binding of Alexa 488-labeled fibrinogen to purified platelets from control (open symbols) and an affected horse (filled circles) was measured at the indicated time points after activation with  $\alpha$ -thrombin. Fluorescence values were normalized to the maximum control value. (B) Western blot of  $\alpha$ IIb and  $\beta$ 3 integrin subunits in resting platelet lysates from a normal (C) and an affected (A) horse; representative of 3 independent experiments. (C) Specific binding of Alexa 488-labeled fibrinogen in response to ADP for normal (open circles) and AET platelets (filled circles). Data are means  $\pm$  SD for 3 control horses and 3 trials with an affected horse.

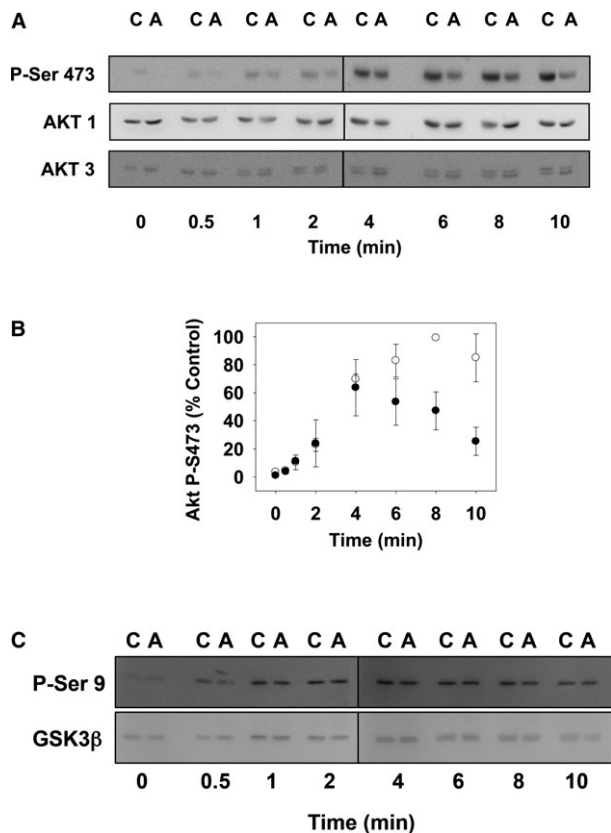
**Table 1.** Fibrinogen binding parameters.

	$B_{max}$ (MFI)	$K_d$ ( $\mu$ g/mL)	$R^2$
Control	$6,392 \pm 833$	$211 \pm 54$	0.99
AET	$4,338 \pm 852$	$182 \pm 65$	0.98
P-value	.072	.545	

AET, atypical equine thrombasthenia.



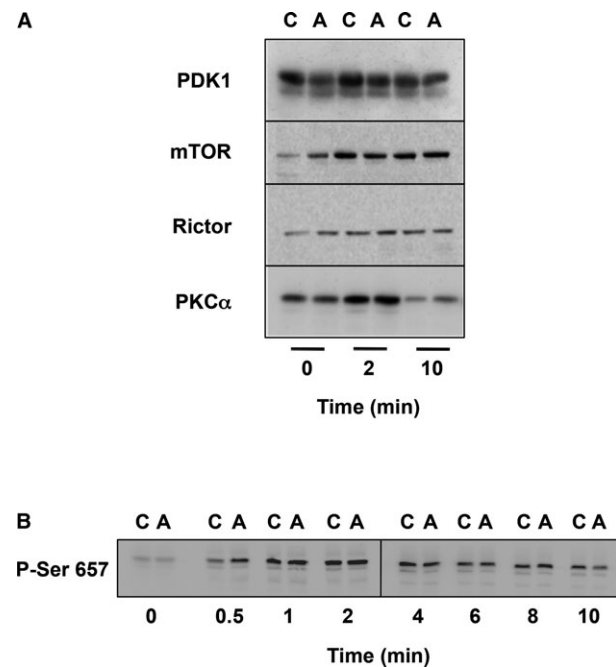
**Fig 2.** Reduced  $\alpha$ -granule and normal dense granule release by atypical equine thrombasthenia (AET) platelets. (A) Western blots of Factor V from total platelet lysates and supernatants of resting or thrombin activated platelets. C and A indicate control and AET platelet samples, respectively. Blots represent 3 independent trials. (B) Mepacrine retention by control (open circles) and AET platelets (filled circles) following activation with the indicated concentration of  $\alpha$ -thrombin. Data are means  $\pm$  SD,  $n = 3$ . For resting platelets, relative fluorescence intensities were  $30.3 \pm 1.7$  (control) and  $32.9 \pm 3.4$  (affected).



**Fig 3.** Abnormal Phosphorylation of protein kinase B (AKT) in atypical equine thrombasthenia (AET) platelets after stimulation with  $\alpha$ -thrombin. (A) Western blots of AKT1, AKT3, and phospho-AKT (S473) from resting platelets and after activation ( $\alpha$ -thrombin, 0.1NIH-U/mL) for the indicated times. C and A indicate control and AET platelet samples, respectively. (B) Changes in phospho-AKT (S473), normalized to the control maximum in each trial, for control (open circles) and AET (closed circles) platelets. Data are means  $\pm$  SD,  $n = 3$ . (C) Western blots of phospho-GSK-3 $\beta$  (serine 9) and total GSK-3 $\beta$  from resting platelets and after activation, as described above. Blots represent 3 independent experiments.

remained at this level for the remainder of the time course. In AET platelets, phosphorylation at this site increased normally for the first 4 minutes after activation, but declined beginning at 6 minutes after activation. This progressive decrease in S473 phosphorylation became significant at 8 minutes after activation (Fig 3B,  $P = .003$  and  $.007$  at 8 and 10 minutes after activation, respectively). We were unable to directly measure AKT phosphorylation at threonine 308 (T308) in equine platelets, a second site regulating AKT function. We detected no difference in phosphorylation of glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) at serine 9, a specific downstream target of AKT phosphorylated on T308, between platelets from control and AET horses after thrombin activation (Fig 3C).

We also found the active, constitutively phosphorylated form of phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates AKT at T308, at equivalent levels in normal and AET platelets before and at 2 and 10 minutes after activation with  $\alpha$ -thrombin

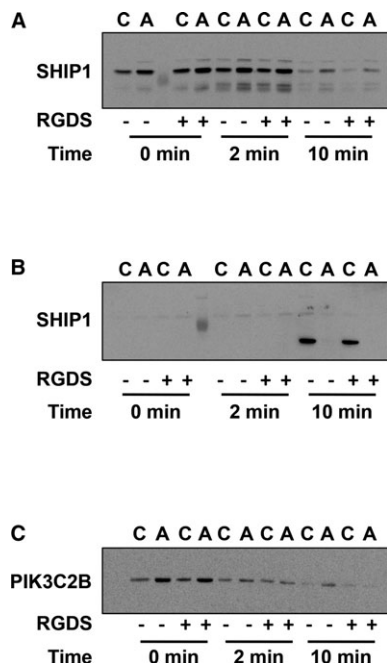


**Fig 4.** Normal expression and function of phosphoinositide-dependent kinase 1 (PDK1) and the mTOR complex 2 (mTORC2) complex in atypical equine thrombasthenia (AET) platelets after stimulation with  $\alpha$ -thrombin. (A) Representative Western blots of total PDK1, rictor, mTOR, protein kinase C (PKC) $\alpha$  from resting platelets and after activation; activation per Fig 3. (B) Changes in phospho-PKC $\alpha$  (serine 657) from resting platelets and after activation; activation per Figure 3. C and A indicate control and AET platelet samples, respectively; blots represent 3 independent trials.

(Fig 4A). Mammalian target of rapamycin (mTOR) phosphorylates AKT on S473, and was present at comparable levels in platelets from normal and affected horses at these time points. This also was observed for rictor, a component of mTOR complex 2 (mTORC2) which provides substrate specificity for AKT.<sup>14</sup> Protein kinase C $\alpha$  (PKC $\alpha$ ), which is phosphorylated by mTORC2 at serine 657,<sup>15,16</sup> was present and phosphorylated identically in resting platelets from all horses and throughout the time course after activation with  $\alpha$ -thrombin (Fig 4B).

SH2-containing inositol-5'-phosphatase 1 (SHIP1) (145 kDa) was detected at comparable levels in the TX-100 soluble fractions of platelets from both affected and control horses before activation with thrombin and at 2 minutes after activation (Fig 5A), but was not apparent in the TX-100 insoluble/cytoskeletal fraction of platelets from either group of horses before activation (Fig 5B). At 10 minutes after activation, SHIP1 was decreased in the TX-100 soluble fractions from platelets of all horses, but more extensively from control platelets. There was a parallel increase in a lower molecular weight isoform of SHIP1 (110 kDa) in the TX-100 insoluble, cytoskeletal fraction of normal platelets at this time point, but not AET platelets.

A second enzyme, PIK3C2B, capable of producing phosphatidylinositol 3',4'-bisphosphate (PI(3,4)P<sub>2</sub>), was



**Fig 5.** Reduced SH2-containing inositol-5'-phosphatase 1 (SHIP1) and normal PIK3C2B levels in atypical equine thrombasthenia (AET) platelets after stimulation with  $\alpha$ -thrombin. (A) Representative Western blots of total SHIP1 in the TX-100 soluble fractions; (B) total SHIP1 in the TX-100 insoluble fraction; and (C) phosphatidylinositol-4-phosphate 3-kinase, class 2 $\beta$  (PIK3C2B) in the TX-100 soluble fractions from control and AET platelets, with indicated time points relevant to activation with  $\alpha$ -thrombin as described in Fig 3. C and A indicate control and AET platelet samples, respectively; blots represent 3 independent trials.

detected in greater amounts in the TX-100 soluble fractions of AET platelets than from normal platelets at all time points (Fig 5C). Treatment of AET platelets with RGDS reduced the PIK3C2B level in this fraction at 10 minutes after activation with  $\alpha$ -thrombin, but this was not observed for control platelets. Phosphatidylinositol-4-phosphate 3-kinase, class 2b (PIK3C2B) was not detectable in the TX-100 insoluble fractions from control or affected platelets at any time point (data not shown).

## Discussion

Here, we described the molecular pathophysiology associated with platelets from Thoroughbred horses with AET (Fig 6). The index case was presented at 3.5 years of age for severe protracted bleeding over a 3-week period in response to pin-firing. During the examination, a template bleeding time of 120 minutes was measured for the index case.<sup>8</sup> Before pin-firing, the index case had no history of bleeding, but subsequently had several hemorrhagic incidents. An offspring with the same platelet dysfunction as the index case, was found to have a normal template bleeding time at less than 1 year of age (Dr Gary Magdesian personal communication). It should be noted that bleeding time in horses have been documented to be extremely variable

and not a quantitative metric of platelet function.<sup>17</sup> However, the bleeding time measured in the affected offspring is consistent with the lack of early hemorrhagic incidents reported for the index case. At 8 years of age, the affected offspring was presented for prolonged and marked hemorrhage with extensive hematoma formation after blunt trauma, which is also consistent with the presentation of the index case.

Compared to controls, platelets from these horses bind markedly less fibrinogen in response to thrombin, despite normal levels of the  $\alpha$ IIB- $\beta$ 3 integrin in platelet lysates and normal fibrinogen binding in response to ADP. The AET platelets also aggregate normally in response to ADP.<sup>9</sup> These features distinguish AET from GT in horses, in which platelets neither express the  $\alpha$ IIB- $\beta$ 3 integrin nor aggregate in response to ADP.<sup>1</sup>

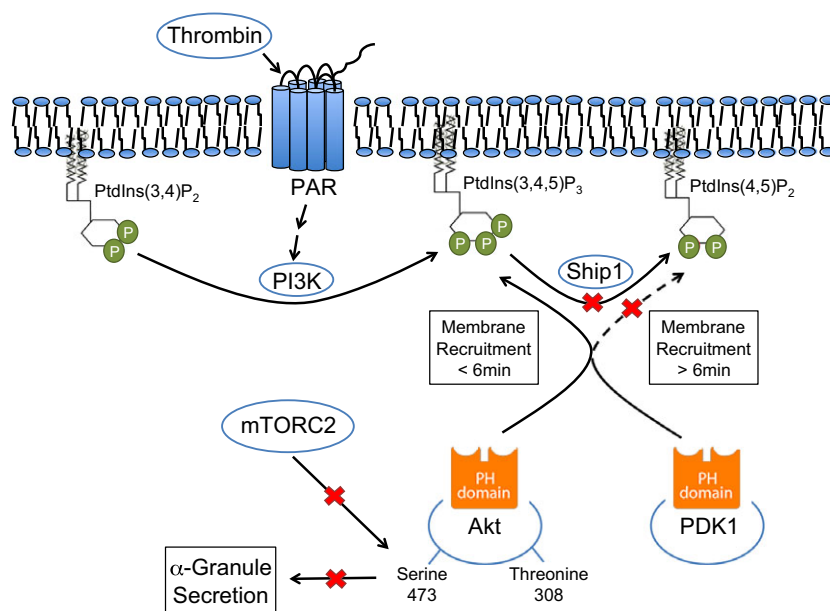
Normal aggregation of AET platelets in response to ADP also is significant because integrin activation by both ADP and thrombin occurs through sequential activation of Gq and Phospholipase-C $\beta$  (PLC $\beta$ ).<sup>18</sup> Therefore, "outside-in" signaling leading to integrin activation and conformational change to the high affinity state is likely to occur normally in the AET platelets irrespective of agonist. The normal kinetics of fibrinogen binding we observed for AET platelets in response to thrombin is consistent with this interpretation.

Platelets secrete dense granules rapidly, whereas the rate of  $\alpha$ -granule secretion depends on the activating stimulus. Dense granule secretion depends on signaling through Gq and PLC $\beta$ .<sup>18,19</sup> The normal release of these granules from AET platelets is consistent with our observation of normal fibrinogen binding in response to ADP.

Factor V is released by  $\alpha$ -granules and is a particularly important modulator of coagulation, because it accelerates Factor Xa-mediated thrombin activation and subsequently, fibrinogen polymerization.<sup>20</sup> Under conditions similar to those used in this study, Factor V has been shown to be slowly released over approximately 10 minutes.<sup>21</sup> The observation that AET platelets secrete significantly less Factor V than normal platelets, despite having normal levels of Factor V, is important for 2 reasons. First, this result provides evidence that our previous report of reduced thrombin activation and fibrinogen polymerization is consistent with reduced Factor V secretion.<sup>9</sup> Second, in combination with the observation that dense granule secretion occurs normally, this result suggests that late signaling events after platelet activation are responsible for the defective Factor V secretion.

Our finding that AET platelets do not sustain phosphorylation of AKT at S473 beginning at 6 minutes after activation, despite normal levels of AKT1 and 3, is consistent with this hypothesis. Deletion of either of these isoforms from platelets markedly reduces fibrinogen binding and secretion of both dense and  $\alpha$ -granules in response to stimulation with thrombin or other agonists of protease activated receptors.<sup>11,22,23</sup>

Serine 473 (S473) is phosphorylated by mTORC2. Normal levels of the mTORC2 components rictor and mTOR, as well as normal phosphorylation of the



**Fig 6.** A model of phosphoinositide signaling pathways leading to  $\alpha$ -granule secretion. Normal platelet signaling after thrombin stimulation includes the production of PI(3,4,5)P<sub>3</sub> by Phosphatidylinositol 3-Kinase. Binding of PH domains in protein kinase B (AKT) and phosphoinositide-dependent kinase 1 (PDK1) to PI(3,4,5)P<sub>3</sub> leads to membrane recruitment and activation. Dephosphorylation of PI(3,4,5)P<sub>3</sub> by SH2-containing inositol-5'-phosphatase 1 (SHIP1) to form PI(3,4)P<sub>2</sub> maintains activation of these kinases at later time points after activation. S473 of AKT is phosphorylated by mTOR complex 2 (mTORC2). "X"s indicate defective signaling events in AET platelets.

mTORC2 target serine 657 of PKC $\alpha$  after activation, provide evidence that this complex functions normally in AET platelets.<sup>15,16</sup> Although recent studies have shown that pharmacological inhibition of mTORC2 does not inhibit aggregation of washed platelets, the role of this complex in fibrinogen binding and  $\alpha$ -granule secretion remains undefined.<sup>14,24</sup> Glycogen synthase kinase (GSK-3 $\beta$ ) phosphorylation also occurs normally after the pharmacological inhibition of mTORC2.

Phosphorylation of GSK-3 isoforms negatively modulates both platelet aggregation *in vitro* and thrombus formation *in vivo*.<sup>25,26</sup> Genetic deletion of PDK1 from platelets prevents phosphorylation at T308 and subsequent phosphorylation of GSK-3 $\beta$ , without affecting fibrinogen binding to platelets after thrombin stimulation.<sup>27,28</sup> As we could not detect phosphorylation of AKT at T308 in equine platelets and normal levels of PDK1 were present in AET platelets, we used phosphorylation of GSK-3 $\beta$  as a surrogate for phosphorylation of AKT at T308 and found no differences between AET and control platelets after activation with thrombin.

Phosphorylation of AKT at both T308 and S473 requires translocation of AKT and PDK1 from the cytosol to the inner leaflet of the plasma membrane. This movement depends on the binding of the pleckstrin homology (PH) domains of both proteins to either phosphatidylinositol 3',4',5'-trisphosphate (PI(3,4,5)P<sub>3</sub>) or PI(3,4)P<sub>2</sub>.<sup>29</sup> Phosphatidylinositol 3',4',5'-trisphosphate (PI(3,4,5)P<sub>3</sub>) is rapidly produced after activation of platelets by thrombin; reaches a peak after approximately 2 minutes; and then, declines over the subsequent 4–6 minutes.<sup>30</sup> Phosphatidylinositol 3',4'-bisphosphate (PI(3,4)P<sub>2</sub>) production begins approximately 6 minutes after thrombin stimulation and

reaches a steady state that is maintained for at least 6 minutes. The overlap of these 2 waves of phosphoinositide production results in continuous phosphorylation of AKT at S473 in platelets after thrombin stimulation. In AET platelets, decreased S473 phosphorylation at late time points is consistent with the loss of the second phase of phosphoinositide production.

Two enzymes have been postulated to regulate the production of PI(3,4)P<sub>2</sub>: PIK3C2B and SHIP1.<sup>31,32</sup> As PIK3C2B was present in platelets from all horses and was present at normal levels in platelets from horses with AET at all times after activation with thrombin, we focused on SHIP1, which has been associated with 70% of the PI(3,4)P<sub>2</sub> production after activation with thrombin.<sup>33</sup>

Solubilization of platelets with TX-100 leads to the separation of the insoluble actin cytoskeleton from the soluble platelet components. In addition to the actin cytoskeleton, the TX-100 insoluble/cytoskeletal fraction is composed of associated proteins that redistribute from the cytosol and membrane, including the  $\alpha$ IIB- $\beta$ 3 integrin. In human platelets, the 145 kDa isoform of SHIP1 redistributes to the cytoskeletal fraction in a manner dependent on activation of the  $\alpha$ IIB- $\beta$ 3 integrin.<sup>32</sup> In normal equine platelets this process occurs within 10 minutes after thrombin activation, along with production of a 110 kDa SHIP1 isoform. This isoform, which retains catalytic activity, can be produced by calpain-mediated, C-terminal proteolysis.<sup>34</sup> However, the loss of the 145 kDa SHIP1 isoform in both TX-100 soluble and insoluble fractions, along with the absence of 110 kDa isoform in AET platelets after activation with thrombin, is consistent with the observed reduction in AKT phosphorylation at S473.

Deletion of SHIP1 from murine platelets results in a phenotype with several features similar to those we have observed in AET platelets.<sup>35</sup> SH2-containing inositol-5'-phosphatase 1<sup>-/-</sup> (SHIP1<sup>-/-</sup>) platelets have reduced P-selectin expression, which is consistent with reduced  $\alpha$ -granule secretion. In addition, under conditions where  $\alpha$ -granule secretion is minimized, these platelets bind normal amounts of fibrinogen, consistent with normal "inside-out" signaling through the  $\alpha$ IIB- $\beta$ 3 integrin. Finally, SHIP1<sup>-/-</sup> platelets show markedly decreased aggregation in response to collagen, which is another hallmark of platelets from horses with AET.<sup>8,9</sup>

Consistent with previous reports, the observations presented here show that decreased fibrinogen binding by AET platelets is associated with decreased Factor V secretion and not defects in integrin  $\alpha$ IIB- $\beta$ 3 function. As Factor V is secreted from platelet  $\alpha$ -granules and phosphorylation of AKT at S473 is needed for normal  $\alpha$ -granule secretion, these data also provide preliminary evidence that the defect in AET platelets results from aberrant signaling through the AKT pathway. Currently, whole genome analysis of horses with AET is underway with the intent to determine the underlying molecular defect associated with this platelet dysfunction and to provide a tool for identifying affected horses.

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### Footnotes

- <sup>a</sup> Unless specifically indicated, all reagents were purchased from Sigma-Aldrich, St. Louis, MO
- <sup>b</sup> Calbiochem, San Diego, CA
- <sup>c</sup> A<sup>C</sup>•T diff Analyzer, Beckman-Coulter, Miami, FL
- <sup>d</sup> Alexa 488-labeled fibrinogen, Invitrogen, Carlsbad, CA
- <sup>e</sup> ADP, Chronolog, Havertown, PA
- <sup>f</sup> Bovine  $\alpha$ -thrombin, Hematologic Technologies, Essex Junction, VT
- <sup>g</sup> FC500, Beckman-Coulter, Miami, FL
- <sup>h</sup> Soybean trypsin inhibitor, leupeptin, AEBSF, and pepstatin, EMD Biosciences, Rockland, MA
- <sup>i</sup> Dithiothreitol, Thermo Scientific, Waltham, MA
- <sup>j</sup> All reagents for SDS-PAGE and nitrocellulose, Bio-Rad, Hercules, CA
- <sup>k</sup> Factor V (V-20) and SH2-containing inositol-5'-phosphatase 1 (SHIP1) (V-19) Santa Cruz Biotechnology, Santa Cruz, CA. phosphatidylinositol-4-phosphate 3-kinase, class 2 $\beta$  (PIK3C2B) (M02), Abgent, San Diego, CA. All other antibodies, Cell Signaling Technologies, Danvers, MA. AKT-1 (C73H10) and AKT-3 (62A8)
- <sup>l</sup> SuperSignal West Femto Western Blotting Substrate, Thermo Scientific, Waltham, MA
- <sup>m</sup> ChemiDoc-It System, UVP, Upland, CA
- <sup>n</sup> SigmaStat, Systat Software, San Jose, CA

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*Off-label Antimicrobial Declaration* Authors declare no off-label use of antimicrobials.

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