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In vitro phosphorylation of von Willebrand factor by FAM20c enhances its ability to support platelet adhesion.

Qi Da[†,*], **Hyojeong Han**[*,¶], **Christian Valladolid**[*,Φ], **María Fernández**[†,*], **Tanvir Khatlani**[†,*,‡], **Subhashree Pradhan**[†,*], **Jennifer Nolasco**[†,*], **Risë K. Matsunami**[§], **David A Engler**[§], **Miguel A. Cruz**[†,*,¶,Φ], and **K. Vinod Vijayan**[†,*,¶,Φ]

[†]Department of Medicine, Houston, TX 77030, U.S.A.

[¶]Department of Pediatrics, Houston, TX 77030, U.S.A.

[Φ]Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, U.S.A.

[*]Center for Translational Research on Inflammatory Diseases (CTRID), Michael E. DeBakey Veterans Affairs Medical Center (MEDVAMC), Houston, TX 77030, U.S.A.

[§]Department of Houston Methodist Hospital Research Institute, Houston, TX 77030, U.S.A.

[‡]Present address: King Abdullah International Medical Research Center (KAIMRC), Ministry of National Guard Health Affairs, Riyadh, KSA.

Abstract

Background: von Willebrand factor (VWF) mediates platelet adhesion and contributes to hemostasis at sites of vascular injury as well as to arterial thrombosis. The A1A2A3 domains of VWF contain important sites that differentially participate in supporting platelet adhesion. FAM20c (family with sequence similarity 20, member C) has emerged as a serine/threonine kinase, which phosphorylates extracellular proteins containing the S-X-E/pS motifs that are also found within the VWF A domains. This is of interest because we and others have shown that structural modifications within these A domains influence the ability of VWF to support platelet adhesion.

Objective: We assessed if VWF A domains can be phosphorylated and the functional consequence of phosphorylated VWF.

Results: Here, we show that FAM20c phosphorylated purified plasma VWF, VWF A1A2A3 protein, isolated A2 domain, but not A1 and A3 domain proteins *in vitro*. FAM20c phosphorylated isolated A2 domain at S1517 and S1613 within the S-X-E recognition motif, with S1613 being the major phosphorylation site. Mass spectrometry analysis of purified plasma VWF from healthy

Address correspondence to: K. Vinod Vijayan, Ph.D., or Miguel A. Cruz, Ph.D. Cardiovascular Research Section, Baylor College of Medicine and MEDVAMC, Rm. 146, Bldg. 109, 2002 Holcombe Blvd, Houston, TX 77030. vvijayan@bcm.edu Telephone: 713-770-1822; miguelc@bcm.edu Telephone: 713-794-7775.

Addendum: QD, HH and CV generated and analyzed the data. TK and SP performed the molecular biology aspect of the studies and generated constructs. MF and JN generated the recombinant VWF proteins. RKM and DAE performed and analyzed mass spectrometric data. KVV and MAC conceived, designed, interpreted the study and wrote the paper. All authors reviewed and edited the final manuscript.

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donors revealed several phosphorylation sites, including the S1613 in A2 domain. VWF A1A2A3 domain protein phosphorylated at S1613 promoted stable platelet adhesion and microthrombi at high shear stress. Lastly, under high shear stress VWF treated with FAM20c and ATP robustly supported platelet adhesion, compared to VWF treated with FAM20c in the absence of ATP.

Conclusion: These outcomes indicate that VWF can be phosphorylated by FAM20c *in vitro*, and this novel post-translational modification enhances the adhesiveness of VWF to platelets.

Keywords

von Willebrand factor; protein kinase; phosphorylation; cell adhesion; blood platelets

Introduction

Protein phosphorylation represents a common mechanism to regulate the structure and function of proteins. Historically, phosphorylation has been extensively studied for intracellular proteins, although many extracellular and secreted proteins are also phosphorylated [1–3]. von Willebrand factor (VWF) is a major secreted multimeric glycoprotein that mediates platelet adhesion and aggregation under high shear stress at the site of vascular injury and also contributes to arterial thrombosis [4]. Post-translational modification of VWF including furin cleavage, glycosylation, oxidation, and sulfation has been described to modulate VWF function [5]. However, studies on potential phosphorylation of VWF have been elusive.

Recently, the atypical kinase family with sequence similarity 20 member c (FAM20C) has been described to phosphorylate more than 100 known secreted phosphoproteins within the Ser -X-Glu/pSer (S-X-E/pS) motifs [6]. Notably, the A domains of VWF, which are biologically important in platelet adhesion, contain several of this S-X-E motifs. We hypothesized that phosphorylation of the VWF A domains by FAM20c impacts its function on platelet adhesion. Our studies indicate that FAM20c phosphorylated S1613 in the A2 domain of VWF and *in vitro* phosphorylation of S1613 in the context of recombinant A1A2A3 domains protein enhanced flow-dependent platelet adhesion. Finally, phosphorylation of VWF by FAM20c *in vitro* resulted in an increased platelet adhesion under high shear conditions.

Materials and methods

Generation of VWF A domain proteins and purification of VWF.

S1517A mutations were introduced into VWF A1A2A3 in pSecTag2b vector [7], while S1517A, S1613A and S1517A/S1613A mutations were introduced into VWF A2 in pQE9 vector [8] by site directed mutagenesis kit (Quikchange II XL; Agilent technologies), using primers listed in (Table 1, Supplemental data). To generate VWF A1A2A3 proteins, pSecTag2b plasmids with wild type A1A2A3 and the A1A2A3 S1517 mutant were transfected into HEK293T cells and stable cell lines generated following selection with Hygromycin B. In some studies, HEK293T cells were transiently transfected with pSecTag2b plasmid containing wild type A1A2A3 and either pcDNA3.1 vector containing FLAG tagged FAM20c or a kinase dead mutant D478A. Wild type and the mutant A1A2A3

proteins were subjected to Ni^{2+} column purification as previously described [7]. The samples were dialyzed in TBS (25 mM Tris-HCl (pH 7.4) and 150 mM NaCl). VWF A2 protein and the corresponding mutants were generated as previously described with a minor modification [8]. Specifically, the solubilized inclusion bodies were diluted in ice-cold refolding buffer (50mM Tris, pH7.4, 1M NaCl, 0.5% Tween-20, 1mM $CaCl_2$) with 1:100 ratio for Ni^{2+} column purification. The eluted proteins were dialyzed in TBS with 0.05% Tween-20 and kept at 0°C. The purity and monomeric state of the recombinant A1A2A3 and A2 proteins were assessed by SDS-PAGE gel and stained with Coomassie Blue. Plasma VWF was purified from healthy donors as described previously [9]. Briefly, fresh plasma was obtained from acid-citrate-dextrose (ACD) blood and centrifuged at 43550 *g* for 30 minutes. The resultant supernatant was applied to a Sepharose CL-4B column at room temperature, and the collected VWF fraction containing void volume was concentrated using Aquacide (Calbiochem), and subsequently dialyzed against TBS.

Generation of FAM20c and its purification.

FAM20c was subcloned into pcDNA3.1 (+) vector with the addition of a FLAG tag to the C-terminal. A kinase-dead mutant was generated by introducing D478A mutation via site-directed mutagenesis. FLAG-tagged FAM20c and D478A mutant was transfected in HEK293T cells and the Flag tagged proteins from the conditioned media was purified as described by Tagliabracci et al. [10].

Phosphorylation of recombinant VWF A domains and purified VWF.

In vitro FAM20c kinase assays to assess phosphorylation of VWF and its fragments was performed using conditions previously described for the phosphorylation of small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) [10]. Briefly, 10 μl of 1 μg/ml of FLAG-tagged FAM20c or D478A mutant was mixed with 30 μl of 50–80 μg/ml of purified VWF, VWF A1A2A3 protein, VWF A domain proteins (VWFA1, VWFA2, VWFA3), VWF A2 mutant proteins (S1517A, S1613A, S1517A/S1613A) in a reaction mixture that contains 50 mM HEPES, pH 7, 10 mM $MnCl_2$ and 1 mM [γ−^{32}P] ATP (specific activity: 100–500 cpm/pmol; Amersham) for 1.5 hours at 30°C. In some studies, 400 units of λ phosphatase (New England Biolab) was added after the kinase reaction and incubated at 30°C for 30 minutes. The reaction was terminated by the addition of 10 μl of 6× SDS loading buffer and boiled at 90°C for 5 minutes. Phosphorylation was visualized by autoradiography of the SDS-PAGE gel separated proteins. Concurrently, similar *in vitro* kinase assays without supplementation of [γ−^{32}P] ATP were performed and the SDS-PAGE gel separated proteins were stained with Coomassie Blue to show the protein loading.

Mass spectrometric analysis.

Please see supplemental data for detailed information. Briefly, A2 protein was subjected to *in vitro* kinase assay using nonradioactive ATP as described above. As a control, similar reactions were performed in the absence of ATP. An aliquot of these reactions was separated on SDS-PAGE and visualized with Coomassie Blue staining. Bands corresponding to the A2 domain protein were excised from gels after *in vitro* kinase assays and subjected individually to in-gel enzymatic digestion with trypsin or other endoproteinases (Asp-N and Glu-C) after reduction and alkylation. Purified VWF isolated from human plasma was

similarly enzymatically digested in solution after reduction and alkylation. Peptides resulting from the enzymatic digests were separated by liquid chromatography (LC) and identified using mass spectrometry (MS). Sites of phosphorylation were identified from the resulting mass spectral data using vendor supplied software, and all sites of phosphorylation were verified using manual inspection of the spectral data from multiple independent enzymatic digests of samples, and with repeated measures across multiple independent LC/MS analyses.

Platelet adhesion studies.

Following the approval from the Institutional Review Board of the Baylor College of Medicine, blood from healthy donors was drawn into 3.8% sodium citrate in a 9:1 ratio. Platelet adhesion studies were performed in a microfluidic Bioflux system (Flexion). Plasma purified VWF (20 μg/mL) or S1517A A1A2A3 protein (40 μg/ml) was incubated with 0.1μg/ml FAM20c in the presence or absence of nonradioactive ATP at 30°C for 2 hours and then immobilized to the Bioflux plate channels. In other studies, Bioflux plate channels were immobilized with A1A2A3 protein that was purified from HEK cells transfected with D478A mutant or FAM20c. The surface was blocked with 3% BSA in TBS prior to perfusion with 10 μM mepacrine labeled whole blood at shear rate of $1500S^{-1}$. Snapshots of adhered platelets were taken along the channel for further analysis. Integrated fluorescent intensity was quantified to represent the amount of adhered platelets as we have performed before [11].

VWF-A2 cleavage assay.

Cleavage assay was performed as we have previously described using recombinant His tagged VWF-A2 protein with modifications [12]. Briefly, A2 protein subjected to *in vitro* FAM20c kinase assay in the presence or absence of ATP was absorbed to Ni-NTA HisSorb strips (Qiagen) as substrate. Microtiter wells were incubated with diluted plasma for 16 hours. Following washing, A2 was detected with human VWF-A2 antibody (R&D Systems, Clone # 210909), which recognized amino acids 1606–1665 C terminus of the cleavage site. Cleavage of A2 by plasma was shown as reduction of VWF-A2 antibody recognition as compared to TBS control.

Statistical analysis.

Results are expressed as mean ± SEM, Student's t test was used to analyze significance of the data.

Results

FAM20c phosphorylates recombinant A1A2A3 and A2 domain proteins of VWF.

Since VWF A domains possess multiple S-X-E motifs, we tested if A1A2A3 protein can be phosphorylated by FAM20c. *In vitro* kinase assays were performed with recombinant FAM20c and purified A1A2A3 protein in the presence of radioactive [γ^{32}P] ATP. FAM20c incorporated ^{32}P from radioactive ATP to A1A2A3 protein (Fig 1A). This reaction also led to the autophosphorylation of FAM20c (Fig.1A). In contrast, the FAM20c inactive mutant (D478A) was unable to phosphorylate A1A2A3 (Fig. 1A). Treatment with λ phosphatase

diminished the phosphorylation of A1A2A3 protein, confirming a phosphorylation event (Fig. 1A). To identify the specific A domain(s) that can be phosphorylated, we performed FAM20c-mediated kinase assays with isolated A1, A2 and A3 domain proteins of VWF. Only the A2 domain of VWF was effectively phosphorylated by FAM20c (Fig. 1B). These studies indicate that under *in vitro conditions*, FAM20c can phosphorylate recombinant A1A2A3 protein and the A2 domain protein of VWF.

Identification of FAM20c phosphorylation sites within the A2 domain of VWF.

To specifically identify the sites of phosphorylation in the A2 domain of VWF, we subjected the recombinant A2 domain to FAM20c-mediated phosphorylation in the presence and absence of non- radioactive ATP and phosphopeptides was evaluated using mass spectrometry. Several isoforms of phosphorylated peptides were observed in the low energy (MS^1) precursor ion scan of the LC/MS^e analysis only in the samples where A2 and FAM20c were incubated in the presence of ATP, but not in samples without ATP (circled as *i-iv* as exemplified from a trypsin digested sample shown in Fig. 2A). These phospho isoforms corresponded to the peptide sequences IGEADFNR**pS**K (circled in i), **pS**KEFMEEVIQR (circled in *ii*), EQAPNLVYMVTGNPA**pS**DEIK and EQAPNLVYoxMVTGNPA**pS**DEIK (circled in *iii*), EQAPNLVYMVTGNPA**pS**DEIKR and EQAPNLVYoxMVTGNPA**pS**DEIKR (circled in *iv*), wherein **pS** refer to phosphorylated serine residues and ox refers to oxidation. As exemplified in the high energy (MS^2) product ion spectra of the LC/MS^E analysis shown (Fig. 2B), derived from the precursor ions found in circles (ii) and (iv) respectively, the presence of these multiple phosphopeptide isoforms all correspond to VWF being phosphorylated on serine 1517 and serine 1613 located within the A2 domain. These two sites of phosphorylation were also confirmed with the Asp-N and Glu-C digested samples independently (data not shown). Importantly, both S1517 and S1613 are contained within the S-X-E motif, which is the consensus site for FAM20c phosphorylation.

To confirm the data obtained from mass spectrometry, we introduced serine to alanine mutation in the A2 protein at either amino acids 1517 (S1517A), 1613 (S1613A), and both 1517 and 1613 (S1517A/S1613A). These mutant proteins were then subjected to FAM20c *in vitro* kinase assays. FAM20c mediated phosphorylation of the A2 domain was slightly decreased in the S1517A mutant, diminished in the S1613A mutant, and abolished in the double S1517A/S1613A mutant (Fig. 2C). These studies indicate that S1517 and S1613 are bona fide sites for FAM20c mediated phosphorylation in the A2 domain, with S1613 being the major phosphorylation site.

VWF purified from plasma of normal subjects is phosphorylated at S1613.

To assess if VWF is phosphorylated endogenously, we purified VWF from the plasma of normal individuals and subjected it to mass spectrometric analysis. These studies revealed that VWF is phosphorylated on serine, threonine and tyrosine residues at multiple sites throughout the molecule (see Table 2; supplemental data). Phosphorylation of residues within the A domains were also identified including the phosphopeptide EQAPNLVYMVTGNPA**pS**DEIK (Fig. 3), which contained the pS1613 site. However no peptides containing phosphate at the S1517 site could be positively identified by our

analyses. These analyses indicate that endogenous VWF is phosphorylated and S1613 in the A2 domain is one of the targets of this post-translational modification.

Phosphorylation of VWF A1A2A3 at S1613 enhances platelet adhesion.

Since FAM20c but not its inactive mutant (D478A) was able to phosphorylate A1A2A3 *in vitro* (Fig. 1A), we transfected HEK 293 cells with A1A2A3 and FAM20c constructs or A1A2A3 and an inactive FAM20c mutant (D478A). A1A2A3 protein purified from the culture supernatant in both conditions (Fig. 4A) was tested for its ability to support platelet adhesion. Compared to the A1A2A3 protein from cells transfected with D478A mutant, A1A1A3 protein obtained from FAM20c transfected cells supported robust platelet adhesion (Figs. 4B and 4C). These studies suggest that cellular conditions which favor A1A2A3 phosphorylation can promote stable platelet adhesion.

Since S1613 in the VWF A2 domain was the major FAM20c site of phosphorylation and phosphorylated S1613 was identified in plasma VWF, we evaluated the functional consequence of S1613 phosphorylation. To overcome the challenges associated with generating VWF phosphorylated only at S1613, we made the VWF A1A2A3 protein variant that can be phosphorylated only at S1613 by FAM20c. To this end, we generated a VWF A1A2A3 protein with a serine to alanine mutation in the A2 protein at amino acid 1517 (A1A2A3 S1517A), (Fig. 5A) and subjected this protein to FAM20c kinase assay in the presence and absence of non-radioactive ATP. FAM20c treatment of A1A2A3 S1517A variant in the presence of ATP will result in an A1A2A3 protein phosphorylated only at S1613 because the 1517 site is blocked and the adjoining A1 and A3 domains were not phosphorylated by FAM20c (Fig 1B).

Perfusion of whole blood at a shear rate of 1500 S^{-1} on S1517A A1A2A3 mutant treated with FAMc20 in the absence of ATP led to platelet adhesion. This observation was similar to studies performed when blood was perfused over wild type A1A2A3 protein. In contrast, A1A2A3 S1517A variant treated with FAM20c and ATP (conditions that enable phosphorylation of S1613) supported stable platelet adhesion and microthrombi (Figs. 5B and 5C). These results suggest that phosphorylation of S1613 in A1A2A3 VWF protein can support stable platelet adhesion.

FAM20c phosphorylates purified VWF and enhances its adhesiveness to platelets.

Next, we tested if VWF purified from plasma can be phosphorylated *in vitro* by FAM20c. Recombinant FAM20c but not its inactive mutant (D478A) in the presence of radioactive [γ^{32}P] ATP phosphorylated VWF. Autophosphorylation of FAM20c was also evident in this assay (Fig. 6A). To investigate the functional consequence of the *in vitro* phosphorylation of VWF by FAM20c, we analyzed platelet adhesion to VWF that was previously mixed with FAM20c in the presence and absence of non-radioactive ATP. Platelet adhesion to immobilized VWF in the presence of ATP was significantly increased compared to VWF treated without ATP (Figs. 6B and 6C). These studies indicate that VWF can undergo phosphorylation by FAM20c *in vitro* and this modification can enhance the adhesiveness of VWF to platelets.

Discussion

Our studies demonstrate for the first time that VWF can undergo phosphorylation, and although the phosphorylation is widespread in the VWF molecule as found in plasma by mass spectrometry, we have focused our attention on phosphorylation within the A2 domain. *In vitro* studies revealed that the A2 domain of VWF can be phosphorylated by FAM20c at S1517 and S1613, while endogenous plasma VWF was found to be phosphorylated at S1613. *In vitro* studies with FAM20c phosphorylated VWF and the S1613 phosphorylation in A1A2A3 variant supported the notion that this post-translational modification promotes an enhanced interaction with platelets.

Previous studies have identified *in vitro* phosphorylation of extracellular plasma proteins like fibrinogen, complement protein C3, vitronectin, factor V and protein S by kinases including casein kinase 1, casein kinase 2, protein kinase C and protein kinase A [13–17]. However, these kinases are predominately localized in the cytoplasm and/or nucleus and not likely to encounter extracellular proteins. Recently, FAM20c and vertebrate lonesome kinase (VLK) have emerged as secreted protein kinases that phosphorylate extracellular proteins on serine or tyrosine residues, respectively [10] [18]. Both kinases possess signal peptide which localizes them to the secretory pathway and are also secreted to the extracellular environment. Specifically, FAM20c is localized to the inner lumen of the endoplasmic reticulum (ER)/golgi and phosphorylates proteins with S-X-E/pS motifs that are destined for secretion [10]. A recent comprehensive analysis of the substrates of FAM20c has identified more than 100 secreted phospho-proteins bearing S-X-E/pS motifs that were secreted from HepG2 liver cells [6]. Interestingly, among the proteins that are related to hemostasis, von Willebrand factor A domain containing protein 1 (VWA1) was identified as one of the FAM20c substrates [6]. Our observations that the A domains of VWF undergo endogenous phosphorylation (Table 2, supplemental) and that the isolated A2 domain of VWF can be phosphorylated by FAM20c at S1517 and S1613 are consistent with the above findings. Interestingly, S1613 is solvent exposed in the crystal structure and is conserved across species including human, mice and pig, suggesting a potential role for the serine 1613 phosphorylation

Phosphorylation of VWF by FAM20c had functional consequence since this post translational modification increased platelet adhesion (Fig. 6B and 6C). Using S1517A VWF A1A2A3 mutant, we concluded that phosphorylation of S1613 in A1A2A3 protein supported stable adhesion and promoted the formation of microthrombi (Fig. 5B and 5C). Although S1517 in the A2 domain constitutes an environment that support water molecule and may be important for the A2 structure [19], S1517A mutant by itself did not significantly alter platelet adhesion. We have previously shown that the A2 domain can regulate platelet adhesion by its interaction with A1 domain and platelet vimentin [20]. It is possible that the phosphorylation of S1613 in the A2 domain may weakens its interaction with the A1 domain, provoking the A1 domain to adopt a high affinity binding conformation for GPIbα upon surface adsorption or exposure to high shear stress [21]. Similarly, phosphorylated A2 domain could result in higher binding affinity for the platelet membrane vimentin, thus increasing platelet adhesion. Although the A2 domain contains the site for ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin type motifs) cleavage

of VWF, plasma mediated cleavage of purified A2 protein treated with FAM20c in the presence of ATP was not different from A2 treated with FAM20c in the absence of ATP (Supplementary Fig. 1). Thus, in contrast to the increased cleavage of VWF in a naturally occurring mutation associated with type 2A von Willebrand factor disease (VWD) wherein, serine to proline mutation occurred at 1613 [22], *in vitro* phosphorylation of isolated A2 protein by FAM20c did not impact the cleavage of A2 protein.

Previous studies using high throughput phosphoproteomic methods that captured and analyzed a proportion of the native phosphoproteome from different cell lines and tissues by various phospho-enrichment and mass spectrometry techniques have suggested that VWF could potentially be phosphorylated [23–25]. Our studies reported here indicate for the first time that endogenous plasma VWF is indeed phosphorylated at many sites throughout the molecule, and that the A-domains in particular are heavily phosphorylated. Interestingly, *in vivo* VWF was phosphorylated at S1613 but not at S1517 site (Supplementary Table 2). Although our *in vitro* studies showed that FAM20c can phosphorylate VWF, it likely that *in vivo* phosphorylation of VWF could also be mediated by kinases other than FAM20c because not all phosphopeptides were contained within the SXE motifs. There are intrinsic limitations of this study. FAM20c phosphorylation studies are mostly analyzed in an *in vitro* setting and whether FAM20c from endothelium and/or platelets can phosphorylate VWF *in vivo* remains to be determined. Although, we noted S1613 phosphorylation in the plasma from healthy subjects, it is unclear whether the phosphorylation of VWF occurred in the plasma or the secretory pathway involving ER/golgi. Loss of function mutations in the human *FAM20C* gene results in an osteosclerotic bone dysplasia called Raine syndrome [26;27]. Mice lacking FAM20c have stunted growth with deformities in bone and teeth, reduced bone mineral content, and hypophosphatemic rickets [28;29]. Despite the low prevalence and lethality in humans carrying FAM20c inactivating mutations and the low viability of mice with deletion of FAM20c, these model systems will be needed in future studies to establish the *in vivo* significance of VWF phosphorylation.

In summary, we describe that VWF can undergo phosphorylation by FAM20c *in vitro* and phosphorylation of VWF could be a potentially new regulatory feature of the VWF adhesive function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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Essentials:

Platelet adhesion to von Willebrand factor (VWF) is critical for hemostasis and thrombosis.

Whether VWF can undergo phosphorylation is unknown.

Family with sequence similarity 20 kinase phosphorylates VWF A2 domain at S1517 and S1613.

Phosphorylation of VWF and VWF A1A2A3 domain at S1613 enhances platelet adhesion.

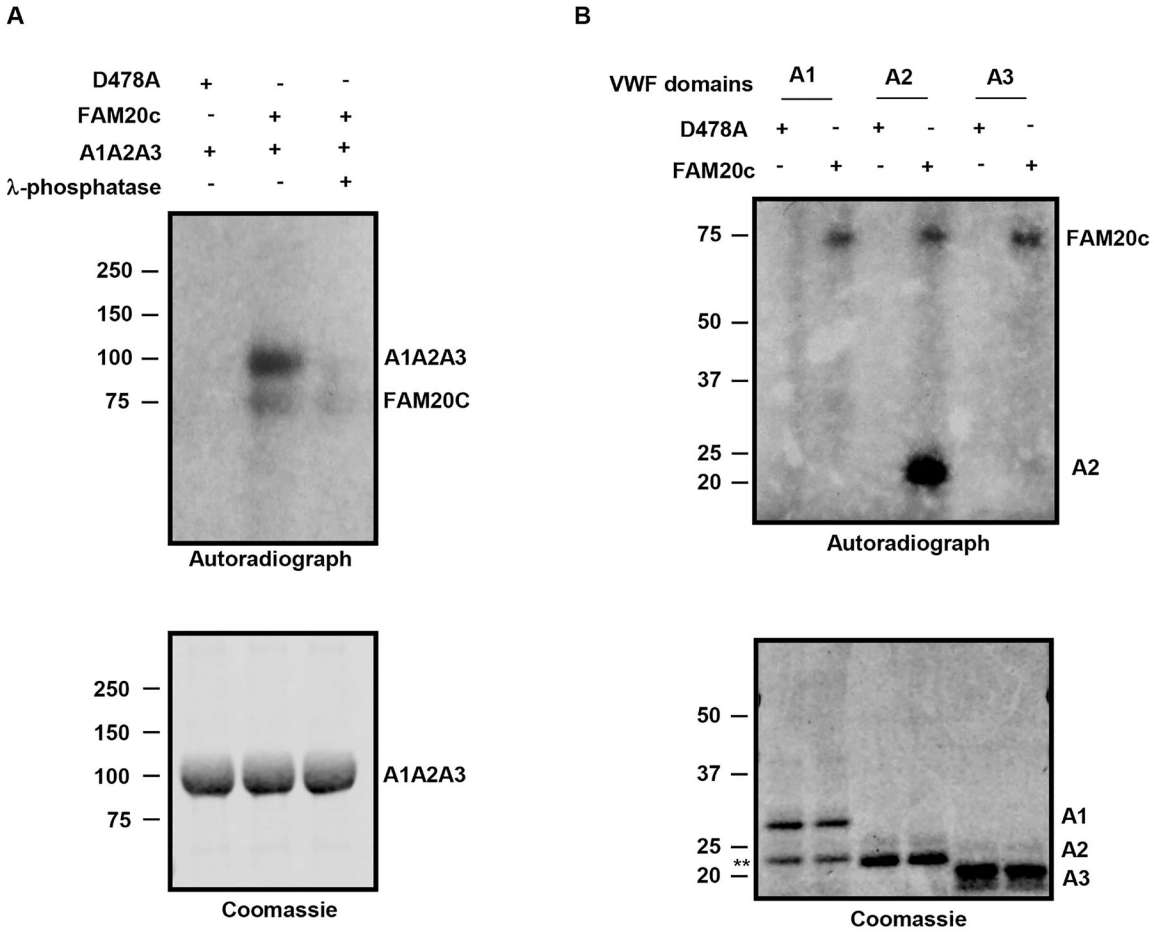
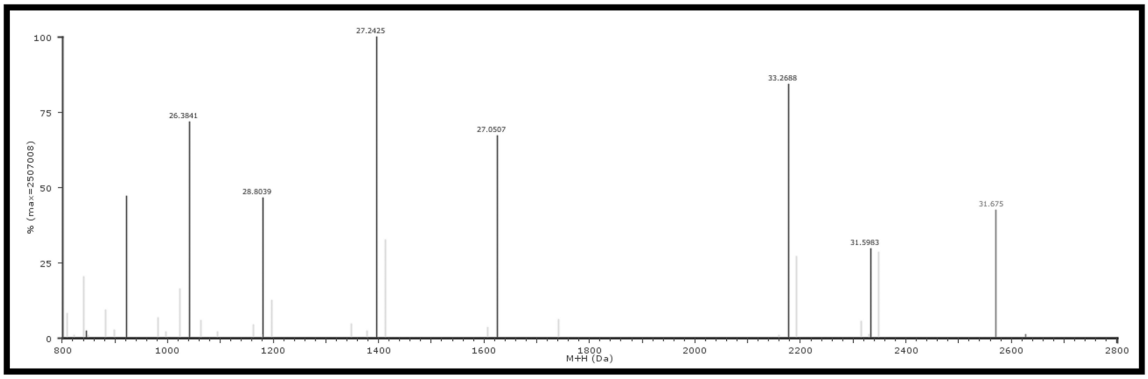


Fig. 1. ***In vitro*** **phosphorylation of VWF A domains by FAM20c.**

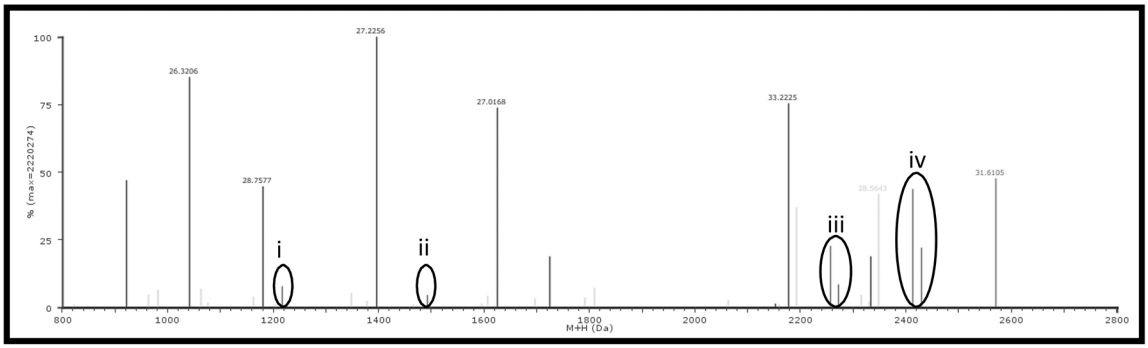
Incorporation of ^{32}P from $\gamma-^{32}P$ ATP to VWF A1A2A3 protein (**A**) and the individual A domains (**B**) by FAM20c but not by the FAM20c kinase dead mutant (D478A). Samples from kinase assays were separated by SDS-PAGE and subjected to autoradiography (upper panel) or visualized by Coomasssie blue staining (lower panel). Degradation product of A1 protein in Figure 1B is shown as **. Phosphorylation of A1A2A3 was blocked by treatment with λ phosphatase. Autophosphorylation of FAM20c in this assay is labelled as FAM20c. Blots are representative of three independent experiments.

2A

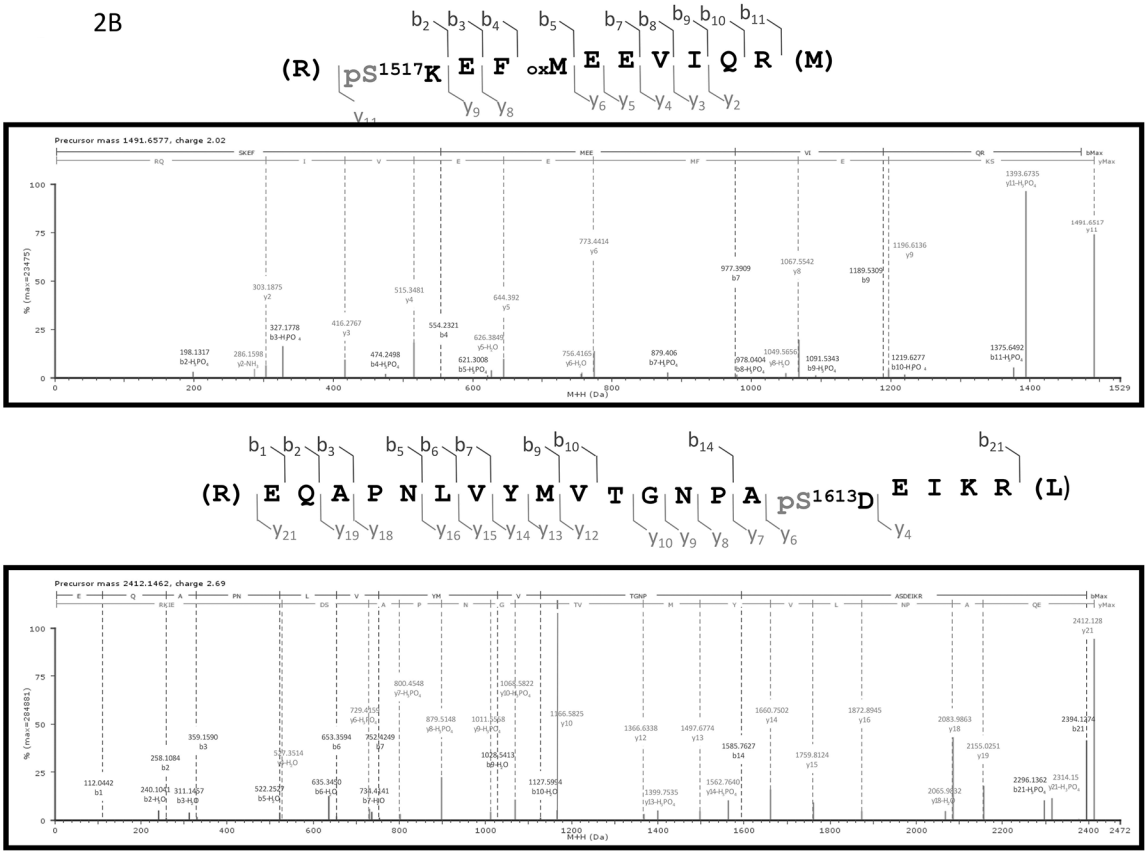
A2 domain -ATP



A2 domain + ATP



2B



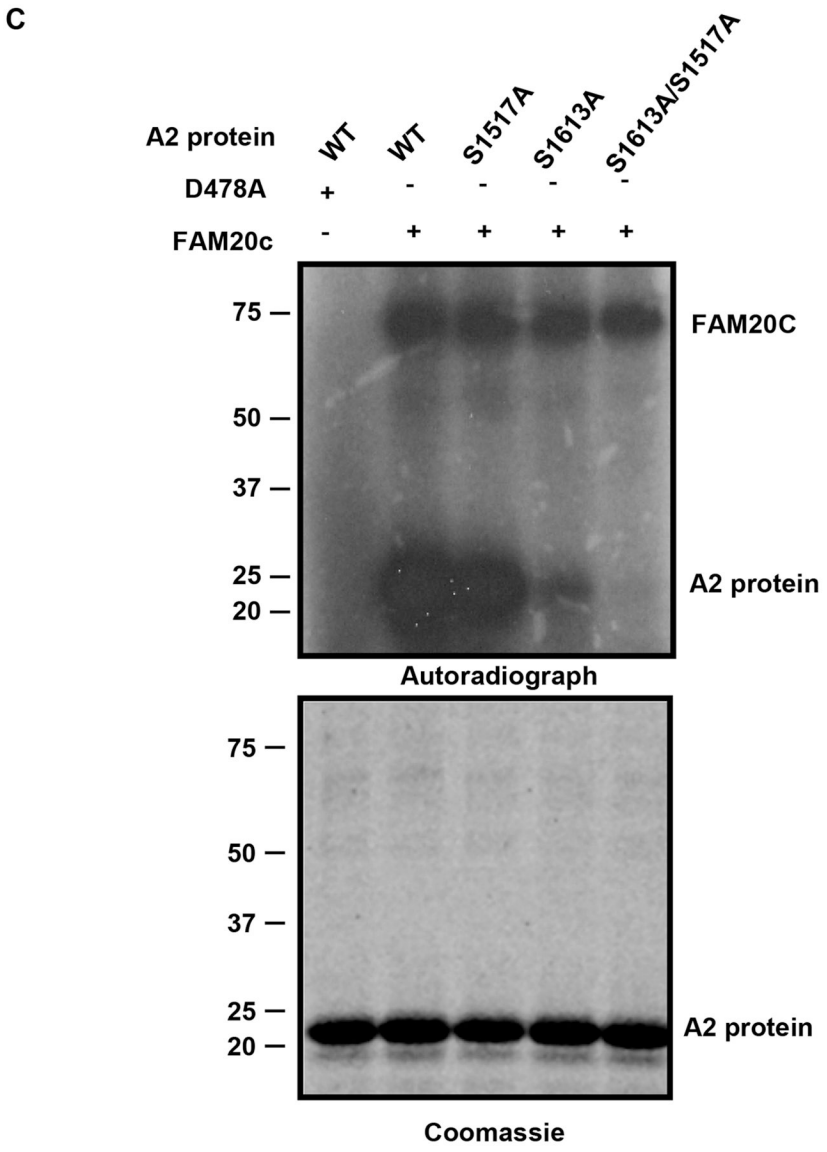


Fig. 2. Identification of S1517 and S1613 in the VWF A2 protein as FAM20c phosphorylation sites.

Mass spectrometric analysis of the A2 protein subjected to FAM20c *in vitro* kinase assays. (**A)** LC/MS^{e} low energy (MS^{1}) precursor ion mass spectra showing A2 domain protein incubated with FAM20C kinase in the absence and presence of ATP. Lock mass corrected and charge-reduced, mass-centroid base peak spectra are shown where x-axis represents M +H^{+} (Da), and y-axis is relative ion intensities; numbers shown on top of spectrum peaks represent LC retention times. Peaks circled in the +ATP spectrum show the presence of phosphorylated peptides not found in the -ATP spectrum, indicating FAM20c-mediated specific phosphorylation. Phosphopeptides in circle ***i*** and circle ***ii*** correspond to phosphopeptide isoforms which include FAM20c-mediated phosphorylation on Ser-1517 and those in circle ***iii*** and ***iv*** to phosphopeptide isoforms which include FAM20c-mediated phosphorylation on Ser-1613. (**B)** LC/MS^{E} base peak high energy (MS^{2}) product ion mass spectra showing all b- and y-ions observed in the MS2 spectrum for two of the

phosphopeptide isoforms circled in ***ii*** and ***iv*** respectively. These MS2 spectra show unequivocally (as summarized in the peptide sequence display shown at the top of each spectra) the site of FAM20c- mediated phosphorylation on the A2 domain protein as being localized to Ser-1517 and Ser-1613. pS refers to site of phosphorylation, and oxM refers to an oxidized methionine residue. **(C)** Incorporation of ^{32}P from γ–^{32}P ATP to wild type (WT) VWF A2 and various A2 mutants by FAM20c and FAM20c kinase dead mutant. Samples were separated by SDS-PAGE and subjected to autoradiography (upper panel) or visualized by Coomassie blue staining (lower panel). Blots are representative of two independent experiments.

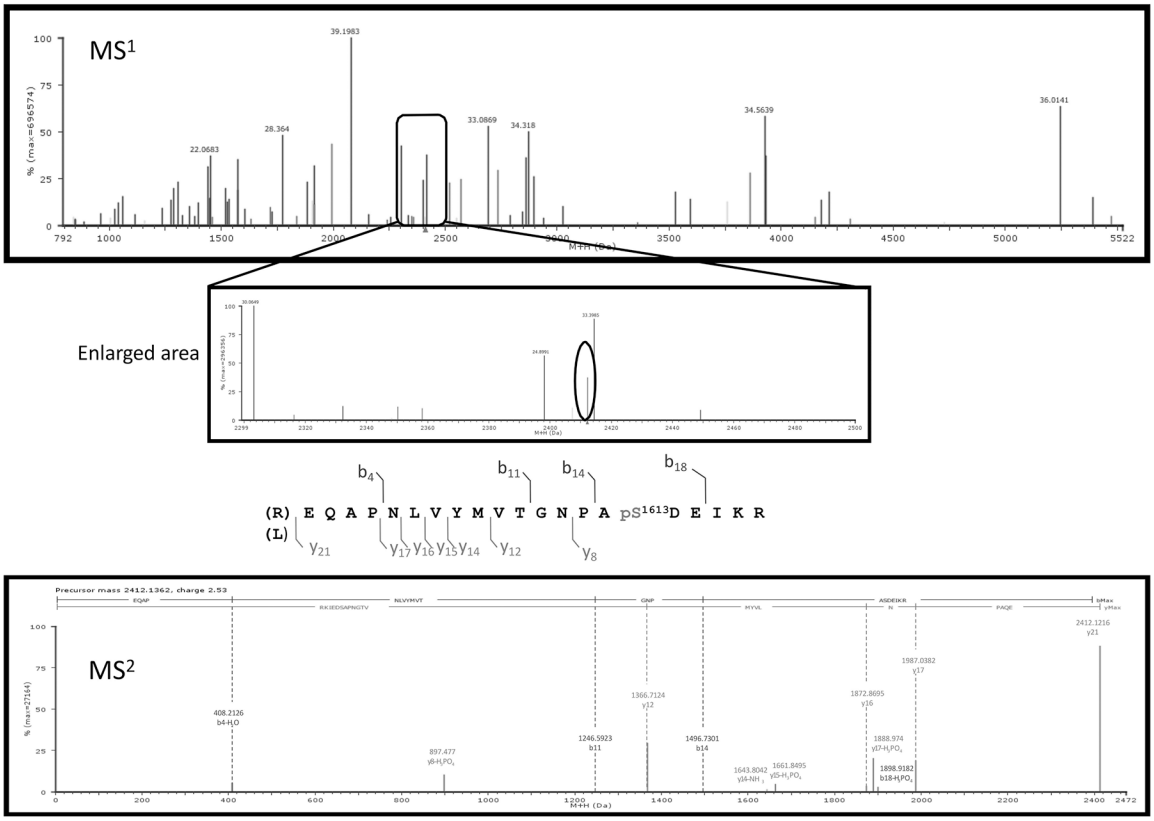


Fig. 3. Identification of S1613 phosphorylation in the purified plasma VWF.

LC/MS spectra showing phosphorylation of the Ser-1613 site within the VWF A2 domain from VWF purified directly from human plasma. LC/MS^{e} low energy (MS^{1}) precursor ion scan of purified VWF after trypsin digestion shown in top spectra, with precursor ion of m/z 2412.1362 shown enlarged and circled within inset, corresponding to peptide sequence EQAPNLVYMVTGNPA**pS**^{1613}DEIKR confirmed by LC/MS^{E} high energy (MS^{2}) sequence analysis shown in lower spectra. Four separate Mass Spectrometry experiments were performed with three different donors.

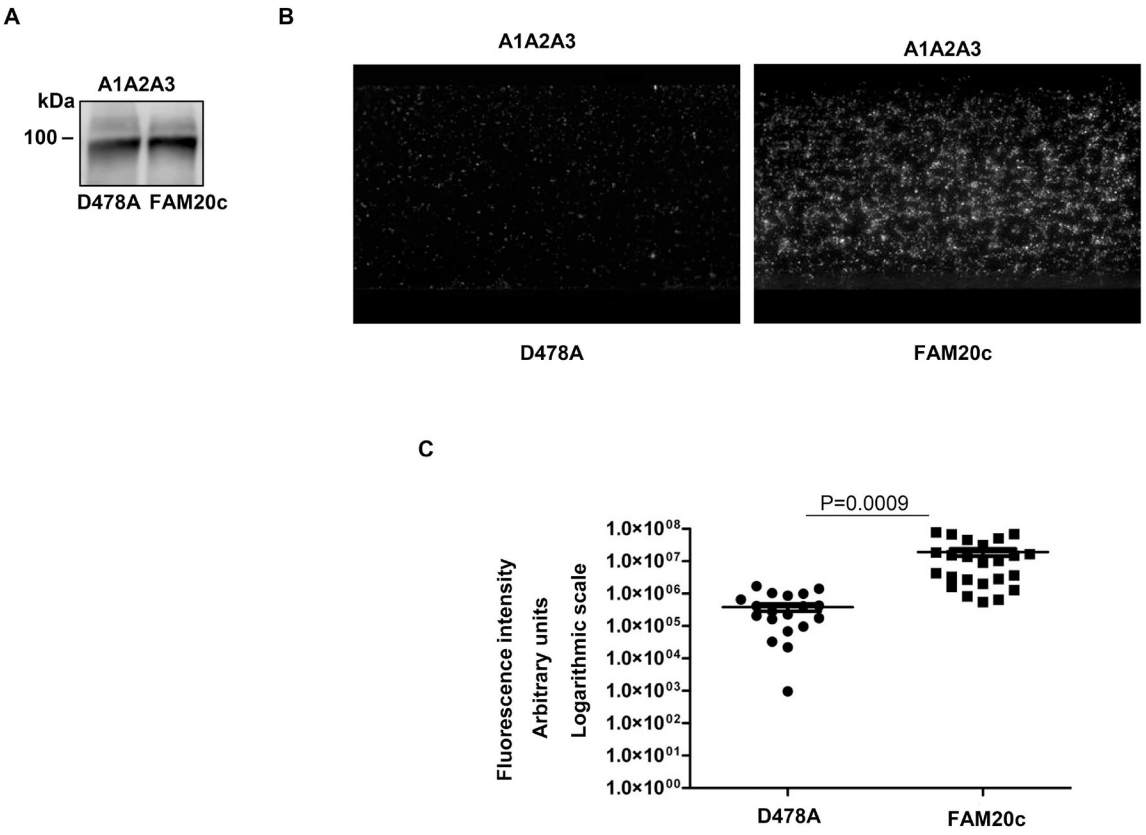


Fig. 4. Increased platelet adhesion to A1A2A3 protein from FAM20c transfected cells

(A) Immunoblotting of A1A2A3 protein purified from HEK 293 cells that are transfected with A1A2A3 and dead FAM20c kinase mutant D478A (D478A) or A1A2A3 and FAM20c (FAM20c) with anti-VWF antibody. (B) Whole blood was perfused over A1A2A3 protein obtained from cells with D478A or FAM20c at a shear rate of 1500 S^{-1} and adhesion visualized in a Bioflux system. (**C**) Quantification of platelet coverage is depicted in a logarithmic scale. Data are obtained from three independent blood donors.

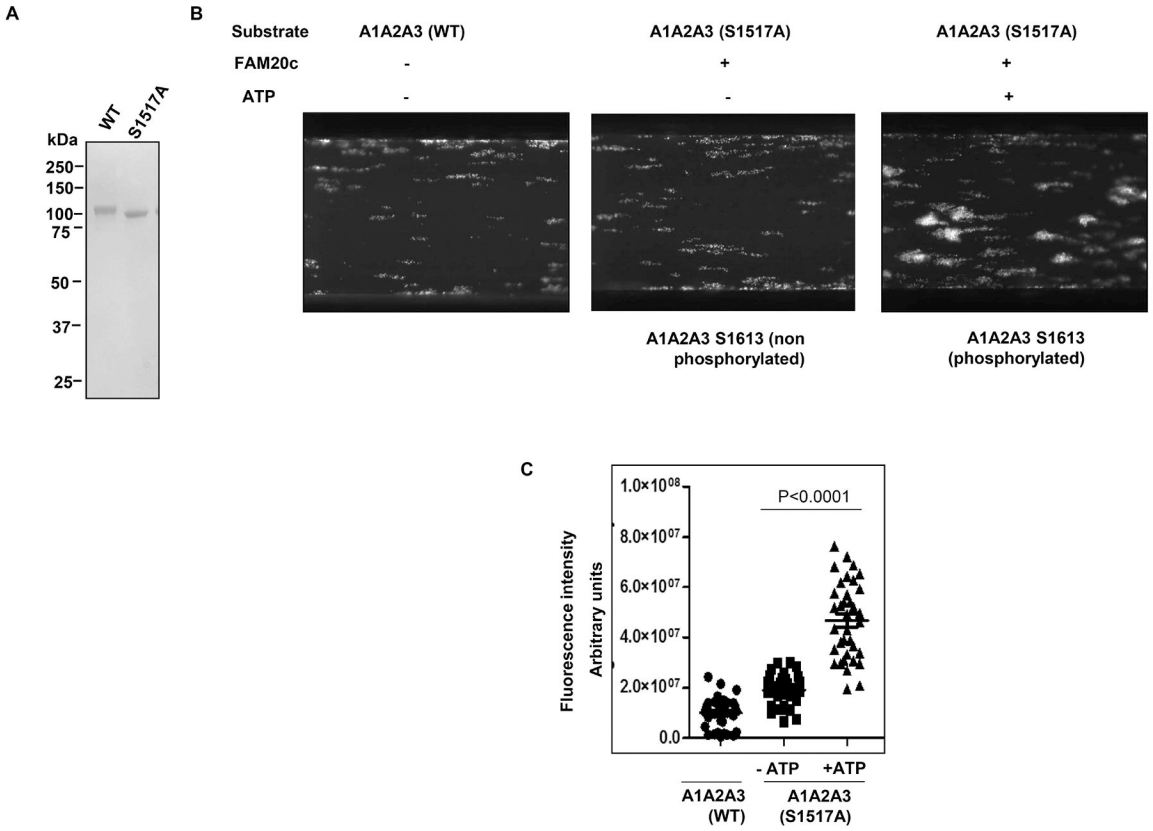


Fig. 5. A1A2A3 protein phosphorylated at S1613 enhances platelet-VWF interaction.

(**A**) Coomassie staining of the purified A1A2A3 wild type (WT) and A1A2A3 S1517A mutant. (**B**) Whole blood was perfused over immobilized A1A2A3 (WT), A1A2A3 (S1517A) mutant that was previously incubated with FAM20c in the presence of ATP (S1613 phosphorylated) and absence of ATP (S1613 not phosphorylated) at a shear rate of 1500 S^{-1} and adhesion visualized in a Bioflux system. (**C**) Quantification of platelet coverage and stable microthrombi. Data are obtained from six independent blood donors.

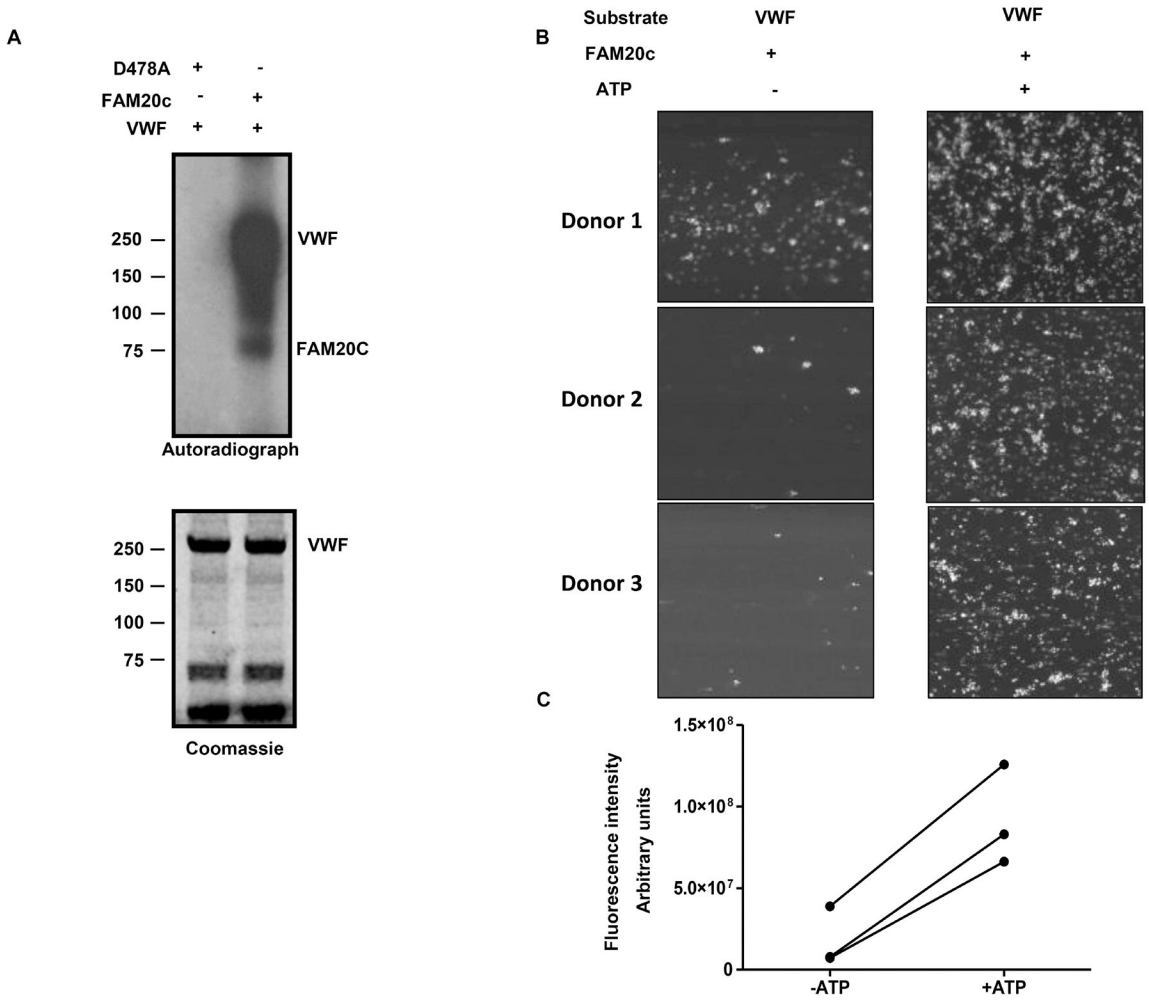


Fig. 6. *In vitro* phosphorylation of VWF supports stable platelet adhesion.

(A) Incorporation of ^{32}P from γ–^{32}P ATP to plasma purified VWF by FAM20c but not by D478A FAM20c kinase dead mutant (D478A). Samples were separated by SDS-PAGE and subjected to autoradiography (upper panel) or visualized by Coomasssie blue staining (lower panel). Autophosphorylation of FAM20c is indicated as FAM20c. Blots are representative of 3 independent experiments. (B) Whole blood from three healthy donors was perfused over a surface coated with purified VWF treated with FAM20c in the presence or absence of non-radioactive ATP at 1500s^{-1} shear rate. (C) Quantification of platelet adhesion.